

**VITAMIN D DEFICIENCY CAUSES MUSCULOSKELETAL HYPERSENSITIVITY:
THE ROLE OF NOCICEPTOR HYPERINNERVATION**

BY

C2011

Sarah Elizabeth Tague

Submitted to the graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Peter G. Smith, Ph.D., Chairman

Kenneth McCarson, Ph.D.

Douglas Wright, Ph.D.

Nancy Berman, Ph.D.

Leslie Heckert, Ph.D.

Date defended:_____

The Dissertation Committee for Sarah Elizabeth Tague certifies
that this is the approved version of the following dissertation:

**VITAMIN D DEFICIENCY CAUSES MUSCULOSKELETAL HYPERSENSITIVITY:
THE ROLE OF NOCICEPTOR HYPERINNERVATION**

Committee:

Peter G. Smith, Ph.D., Chairman

Kenneth McCarson, Ph.D.

Douglas Wright, Ph.D.

Nancy Berman, Ph.D.

Leslie Heckert, Ph.D.

Date approved:_____

ABSTRACT

Clinical studies link vitamin D deficiency and musculoskeletal pain, both of which occur more frequently in women. However, a causal relationship has been difficult to establish and it is not clear whether vitamin D metabolites directly influence nociceptors ('pain-sensing' neurons). It was shown here, via immunohistochemistry and western blot, that rat putative nociceptors contain vitamin D receptors (VDRs) and metabolic enzymes, whose expression is regulated by ovarian hormones. In ovariectomized rats a vitamin D deficient diet induces balance deficits and deep tissue mechanical hyperalgesia, concurrent with muscle hyperinnervation by presumed nociceptors. Balance deficits, muscle mechanical hypersensitivity, and hyperinnervation are not corrected by elevated dietary calcium. In primary sensory cultures, VDR is enriched in c-fiber growth cones and regulates neurite outgrowth through VDR rapid response pathways. Therefore, vitamin D metabolites act directly on nociceptive neurons to inhibit axonal sprouting, accounting for hypovitaminosis D-induced muscle hyperinnervation, and possibly contributing to hypersensitivity.

This work is dedicated to my parents, Susan D. and Walter C. Tague, for making my education a priority.
Without their continued support, this work would not have been possible.

ACKNOWLEDGEMENTS

This work was partially funded by the KUMC Biomedical Research Training Program and a NIH, National Institute on Aging, Kirschstein National Research Service Predoctoral Award.

I have had exceptional mentors over my academic career who have helped lay the groundwork for these studies. I learned the basics of working in a lab during my undergraduate education at the University of Kansas in the labs of Dr. Victoria Corbin and Dr. Elizabeth Boyce. During my master's degree studies at the University of Notre Dame, I learned invaluable molecular and cell biology techniques in the lab of Dr. Crislyn D'Souza-Schorey. Most notably, she showed me how to develop and follow through with plausible hypotheses. Finally, my work in Dr. H. Clarke Anderson's lab at the University of Kansas Medical Center introduced me to translational bench-animal-clinical research. While so much of science is focused on the details, he taught me to always think about the larger picture.

During my studies at the University of Kansas Medical Center, I am grateful to have worked with a wonderful and diverse group of scientists. I thank our lab technicians Zhaohui Liao and Elza Kharatyan, who generally keep the lab efficient and organized, allowing me to focus on science. My Ph.D. experience was enhanced by my fellow students, Aritra Bhattacharjee, Gwenaelle Clarke, Tim Donohue, Argenia Doss, and Eva Selfridge. I owe a special thanks to Eva Selfridge for her excellent proofreading skills. The senior lab members Dr. Dora Krizsan-Agbas and Dr. Anuradha Chakrabarty have provided both guidance and friendship. Both Anuradha and Dora taught me dissection techniques, and Anuradha helped teach me surgical procedures, culture techniques, and behavior analysis. Finally I am especially grateful to Gwenaelle Clarke, an amazing friend who also helped me with the muscle compression analysis and sympathetic culture techniques described here.

I owe much gratitude to the wonderful members of the Kansas Intellectual and Developmental Disabilities Research Center, Dr. Don Warn, Phil Shafer, Jing Huang, Doug Brownyard, Michelle Winter,

Tina Darrow and Beth Van Luchene. They work hard to keep everything running smoothly. I would like to thank Michelle Winter and Dr. Ken McCarson in particular. Michelle helped with animal surgeries, set up behavioral equipment, and performed the serum 25(OH)D assays described here, and Ken helped me design the behavioral experiments.

Dr. Anthony Norman at the University of California Riverside kindly provided the vitamin D analog, JN, which was used in the culture studies described here. Dr. H. Clarke Anderson provided the muscles from rachitic rats for our preliminary experiments.

The Department of Molecular and Integrative Physiology created a wonderful environment for students, thanks to the chair, Dr. Paul Cheney, the staff, and the Physiology Society.

As sometimes happens with the best laid plans, life gets in the way. I owe my deepest gratitude to my friends and family who took care of me when my studies were interrupted by illness. To my mother and father who drove me to each and every doctor's appointment, brought me groceries, and generally took care of me; to my friends, Merritt Engel, Amanda Dewoody, Gwenaelle Clarke, Eva Selfridge, and Rachel Rempfer who offered emotional support, checked in on me regularly, made me food, and cleaned my apartment; to my lab mates who sent me flowers and were incredibly supportive; and to my mentor, Peter Smith, for his patience and understanding, I don't know what I would have done without all of you.

Finally I would like to extend my deepest gratitude to Dr. Peter Smith, a gifted scientist and outstanding leader. In addition to his scientific guidance, he has taught me much about how to develop leadership qualities, grant writing skills, and lab management strategies. His insights into the details of careers in academia and beyond are invaluable. I am grateful that he encourages individual thought and allowed me to pursue my scientific interests. His breadth of knowledge is impressive and I will continue to admire him for his honesty and strength of character. It has been an honor to work with Dr. Smith.

TABLE OF CONTENTS

ACCEPTANCE PAGE.....	ii
ABSTRACT.....	iii
DEDICATION.....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vii
LIST OF TABLES AND FIGURES.....	viii
CHAPTER 1. GENERAL INTRODUCTION.....	1
TABLE.....	10
FIGURE.....	11
CHAPTER 2. VITAMIN D RECEPTOR AND ENZYME EXPRESSION IN DORSAL ROOT GANGLIA OF ADULT FEMALE RATS: MODULATION BY OVARIAN HORMONES	13
ABSTRACT.....	14
INTRODUCTION	15
MATERIALS AND METHODS.....	16
RESULTS	21
DISCUSSION.....	26
TABLES	34
FIGURES.....	35
CHAPTER 3. VITAMIN D DEFICIENCY PROMOTES SENSORY AXON SPROUTING AND MUSCLE HYPERSENSITIVITY	49
ABSTRACT.....	50
INTRODUCTION	51
MATERIALS AND METHODS.....	52
RESULTS AND DISCUSSION.....	57
TABLES	65
FIGURES.....	67
CHAPTER 4. GENERAL CONCLUSIONS AND DISCUSSION.....	83
REFERENCES	93

LIST OF TABLES AND FIGURES

CHAPTER 1

Table

1. Factors that contribute to both musculoskeletal pain and vitamin D deficiency.....10

Figure

1. Increased peripherin content in muscles from vitamin D deficient rats.....11

CHAPTER 2

Table

1. Quantitative analysis of neuronal markers and effect of ovariectomy.....34

Figures

1. VDR, CYP27B1, and CYP24 proteins are present in adult DRG..... 35
2. Cellular localization of VDR, CYP27B1, and CYP24 in adult DRG.....37
3. Cellular colocalization of VDR with the neural markers peripherin, IB4, and CGRP.....39
4. Cellular colocalization of CYP27B1 with the neural markers peripherin, IB4, and CGRP.....41
5. Cellular colocalization of CYP24 with the neural markers peripherin, IB4, and CGRP.....43
6. Cellular colocalization of VDR with the enzymes CYP27B1 or CYP24.....45
7. DRG expression of VDR and CYP27B1, but not CYP24 is reduced following ovariectomy.....47

CHAPTER 3

Tables

1. Diet, weight, and serum analysis.....65
2. No changes in bone parameters were found in rats fed a vitamin D deficient diet for four weeks.....66

Figures

1. Experimental timeline.....	67
2. Modification of the digital Randall Selitto instrument for muscle compressions.....	69
3. Behavioral comparisons of control, VD-/ +Ca, and VD- rats.....	71
4. Representative images of tibial growth plates from rats after four weeks on control, VD-/ +Ca, or VD- diets.....	73
5. Comparisons of innervation densities of the lateral gastrocnemius muscles of rats after four weeks on control, VD-/ +Ca, or VD- diets.....	75
6. Representative images of the immunoreactive nerves in calf muscle.....	77
7. 1,25(OH) ₂ D ₃ acts directly on c-fiber sensory nerves to regulate axonal growth.....	79
8. Sympathetic neurons express VDRs, but 1,25(OH) ₂ D ₃ has no effect on neurite outgrowth.....	81

CHAPTER 1. GENERAL INTRODUCTION

General Background

Accumulating evidence supports a role for vitamin D in certain types of pain. For example, vitamin D deficiency is associated with chronic musculoskeletal pain and acroparaesthesia (numbness, burning, or tingling in the extremities) (Benson, Wilson et al. 2006; de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991; Heidari, Shirvani et al. 2010; Macfarlane, Palmer et al. 2005; McBeth, Pye et al. 2010; Plotnikoff and Quigley 2003). Up to 93% of patients who report chronic musculoskeletal pain are vitamin D deficient (Plotnikoff and Quigley 2003). Several studies have shown that this pain may be relieved by vitamin D supplementation (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991). However, a direct causal relationship has not been established and potential mechanisms underlying this phenomenon have remained unexplored.

Musculoskeletal pain

Musculoskeletal pain in general has enormous socioeconomic impacts on our society. It can significantly decrease the quality of life for an individual by preventing entry into the workforce and rendering many incapable of completing simple daily activities. The council for disability awareness estimates that in 2006, of the \$79.9 billion in social security disability insurance payouts and \$7.2 billion in long-term disability payouts from private companies, musculoskeletal disorders accounted for 25.7% of all disability claims making it the number one cause for long-term disability (Awareness 2006).

Musculoskeletal pain also substantially affects the load on our health care system. It is estimated that the prevalence of chronic musculoskeletal pain (pain lasting longer than 3 months) in adolescents is between 27.5-36% (De Inocencio 2004) and 35-50% in adults (Bergman 2007). In 10-22% of adults this pain is widespread (Abusdal, Hagen et al. 1997; Bergman, Herrstrom et al. 2001; Croft, Rigby et al. 1993; Hunt, Silman et al. 1999; Schochat and Raspe 2003; White, Speechley et al. 1999; Wolfe, Ross et al. 1995). In addition, 20-40% of all primary health care visits are due to musculoskeletal complaints (Andersson, Ejlertsson et al. 1999; De Inocencio 2004). Despite these statistics the mechanisms and pathology that lead to chronic musculoskeletal pain are poorly understood.

Vitamin D

The secosteroid vitamin D is generally considered a prosteroid hormone and is not technically a vitamin because humans can synthesize it; however, most individuals also acquire vitamin D from natural dietary sources (i.e. fatty fish), fortified foods, or nutritional supplements (Yetley 2008). Synthesis begins upon exposure to UVB light, when 7-dehydroxycholesterol is converted to previtamin D within the keratinocyte cell membrane of the skin (Holick 2005). Previtamin D spontaneously isomerizes into vitamin D during the course of several hours and enters the blood stream through mass action, due to the presence of vitamin D binding proteins within the blood (Holick 2005). Whether synthesized or from nutritional sources, vitamin D is converted in the liver to the main circulating form, 25-hydroxyvitamin D (25(OH)D) (Horst, Reinhardt et al. 2005). 25(OH)D can be further transformed by 25-hydroxyvitamin D 1 α -hydroxylase/cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1) into 1,25-dihydroxyvitamin D (1,25(OH)₂D), which is the most well understood active metabolite (Horst, Reinhardt et al. 2005). Conversion of 25(OH)D into 1,25(OH)₂D occurs largely in kidney, but other cell types express CYP27B1 and can locally convert circulating precursor to active hormone (Jones 2007). Hydroxylation of 1,25(OH)₂D to form 1,24,25-trihydroxyvitamin D by the widely expressed enzyme, 25-hydroxyvitamin D 24-hydroxylase/cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24), is an initial step in the pathway of 1,25(OH)₂D degradation, but CYP24 also produces other vitamin D metabolites (i.e. 24,25-dihydroxyvitamin D), whose actions are just beginning to be understood (Omdahl, Bobrovnikova et al. 2003; Sakaki, Kagawa et al. 2005). CYP27B1 and CYP24 therefore act as opposing forces in regulating the concentration of 1,25(OH)₂D. This active hormone binds to vitamin D receptors (VDRs), which are spatially dynamic and may be localized within the nucleus where they influence gene transcription, or within the cytoplasm and membrane where they modulate ion flux and the generation of second messengers (Mizwicki and Norman 2009).

Vitamin D deficiency is prevalent worldwide. It is thought that the best predictor of vitamin D status is serum 25(OH)D levels, because 1,25(OH)₂D has a relatively short half life and is more tightly

regulated (Jones 2008). The NIH recently revised its standards for serum 25(OH)D levels according to a recent Institute of Medicine committee. Previously anything $>37.5\text{nmol/L}$ was considered sufficient, $<37.5\text{nmol}$ was insufficient, and below 25nmol/L was deficient. Now $>50\text{nmol/L}$ (20ng/ml) is considered sufficient for 97.5% of the population, and $<30\text{nmol/L}$ (12ng/ml) is considered deficient (Institute of Medicine 2010). However, a number of researchers feel that these levels should be adjusted even higher (Dawson-Hughes, Heaney et al. 2005; Hollis 2005). Peak calcium absorption from the intestines occurs when serum 25(OH)D is approximately 80pmol/L (32ng/ml) (Heaney, Dowell et al. 2003) and levels $>80\text{pmol/L}$ may be required to alleviate secondary hyperparathyroidism in the elderly (Vieth, Ladak et al. 2003), so some have suggested that above 80nmol/L (32ng/ml) should be considered replete and below this level should be considered insufficient. Some researchers believe that serum 25(OH)D concentrations below 50nmol/L (20ng/ml) should be considered deficient, because there is a 5-15% increase in parathyroid hormone and higher bone turnover (Chapuy, Preziosi et al. 1997; Holick 2009; Need 2006). When concentrations fall below 25nmol/L (10ng/ml) there is an increased fracture risk (Cummings, Black et al. 1993; Ooms, Roos et al. 1995) and below 12.5nmol/L (5ng/ml) is when overt osteomalacia or rickets becomes apparent (Need 2006). While the data available concerning the upper levels are not clear, serum levels above 125nmol/L (50ng/ml) may begin to raise concerns (Institute of Medicine 2010), however toxicity rarely occurs below 500nmol/L (Heaney 2008). Based on the National Health and Nutrition Examination Survey (NHANES), approximately 70% of the population has serum 25(OH)D concentrations that are less than 80nmol/L (32ng/ml), 30% has levels less than 50nmol/L (20ng/ml), and 5% has levels less than 27.5nmol/L (11ng/ml) (Yetley 2008).

Vitamin D and musculoskeletal pain

A significant proportion of patients who report chronic musculoskeletal pain are vitamin D deficient, with serum 25(OH)D concentrations that are less than 50nmol/L (20ng/ml) (Benson, Wilson et al. 2006; Macfarlane, Palmer et al. 2005; Plotnikoff and Quigley 2003). Depending on the population studied and the cut-off values of 25(OH)D used to determine deficiency, the reported percentage of patients with

musculoskeletal pain that are vitamin D deficient varies from 25-100%. Gloth et al. first described five patients with severe pain triggered by minimal movement or touch. All five patients had 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) levels below 45pmol/L (normal range 50-190pmol/L) and four of the five patients had serum 25(OH)D levels below 50nmol/L (20ng/ml). All patients recovered after vitamin D supplementation (Gloth, Lindsay et al. 1991). A study of Australian Aborigines showed that while 100% of Aborigines with muscle pain had a levels of 25(OH)D below 50nmol/L (20ng/ml), only 12.5% of Aborigines without muscle pain were vitamin D deficient (Benson, Wilson et al. 2006). Of 150 people who reported to a US community clinic with non-specific musculoskeletal pain, 93% had 25(OH)D levels below 50nmol/L (20ng/ml) (Plotnikoff and Quigley 2003). A study in Norway of 276 patients with musculoskeletal pain and 229 controls found that 81.6% of patients with leg pain and 60% with chronic widespread pain were vitamin D deficient (<20ng/ml) compared to only 36.1% of controls. They also show that average serum 25(OH)D levels were significantly lower in patients with leg pain (14.5ng/ml) or widespread pain (20.6ng/ml) than in control patients (33.1ng/ml) (Heidari, Shirvani et al. 2010). Results from a recent European Male Ageing Study analyzing 3075 men age 49-79, found that 25.5% of men with chronic widespread pain had 25(OH)D levels below 37.5nmol/L (15ng/ml), compared to only 18.6% in men with no pain (McBeth, Pye et al. 2010). Conversely a study in Switzerland of asylum seekers with hypovitaminosis D (<21nmol/L) found that up to 91% experienced somatic pain, and 85% reported complete or partial resolution of symptoms after vitamin D treatment (de Torrente de la Jara, Pecoud et al. 2006). In addition to pain, it has also been reported that 59% of Arab women with hypovitaminosis D experience acroparaesthesia. After vitamin D supplementation, 92% displayed improvement and 81% reported a complete remission of symptoms (Glerup and Eriksen 1999). In addition to the growing number of studies linking vitamin D deficiency to musculoskeletal pain, there have also been several conflicting reports, which appear to stem from a focus on vitamin D deficiency and fibromyalgia (Block 2004; de Rezende Pena, Grillo et al. 2010; Tandeter, Grynbaum et al. 2009; Warner and Arnspiger 2008). However, this issue may have been cleared up by Heidari et. al, who recently showed that while low vitamin D levels are linked to chronic widespread pain in general, vitamin D status

is not associated with chronic widespread pain subtype, fibromyalgia (Heidari, Shirvani et al. 2010). All in all there is a significant clinical link between vitamin D deficiencies and non-specific musculoskeletal pain, but an understanding of how vitamin D affects the pain pathways of the nervous system is severely lacking.

Vitamin D and the nervous system

Vitamin D is becoming recognized as an important hormone in nervous system health. VDR is expressed by neurons, oligodendrocytes, and astrocytes within the limbic system, basal ganglia, spinal cord, cerebellum, and cerebral cortex (Baas, Prufer et al. 2000; Eyles, Smith et al. 2005; Glaser, Veenstra et al. 1999; Neveu, Naveilhan et al. 1994; Perez-Fernandez, Alonso et al. 1997; Prufer, Veenstra et al. 1999; Walbert, Jirikowski et al. 2001). Studies have also shown that neurons and glia in the brain express CYP27B1 (Eyles, Smith et al. 2005; Zehnder, Bland et al. 2001), and CYP24 mRNA has been detected in glioma and primary glial cell culture (Naveilhan, Neveu et al. 1993), suggesting that the brain can regulate local 1,25(OH)₂D concentrations. Vitamin D signaling has been linked to cell survival, proliferation, and differentiation, and may be especially important during brain development (Eyles, Brown et al. 2003; Levenson and Figueiroa 2008). In the adult, vitamin D supplementation has been proposed to improve outcomes in multiple sclerosis, Parkinson's disease, and traumatic brain injury (Cekic, Sayeed et al. 2009; Myhr 2009; Newmark and Newmark 2007). Expression of VDR has also been identified in fetal (E12-E21) DRG sensory neurons (Johnson, Grande et al. 1996; Veenstra, Prufer et al. 1998), but it was not established whether expression continues post-natally. Thus, accumulating evidence suggests that vitamin D signaling plays important roles in the nervous system. However, little is known about how vitamin D may affect pain pathways.

Sex differences in musculoskeletal pain and vitamin D deficiency

In all age groups after the age of five, vitamin D deficiency is more prevalent in women than men (Yetley 2008). Women are also twice as likely to develop chronic widespread pain (Bergman 2007).

This has led us to consider a role for ovarian hormones in the regulation of the vitamin D system and the development of pain. While testosterone levels do not appear to affect serum 25(OH)D levels or VDR expression (Tiwari, Gupta et al. 2002), VDR expression is up-regulated by 17 β -estradiol in some cell types, including osteoblasts (Duque, El Abdaimi et al. 2002; Liel, Kraus et al. 1992; Mahonen and Maenpaa 1994), breast cancer cells (Escaleira, Sonohara et al. 1993; Gilad, Bresler et al. 2005), colonocytes and duodenocytes (Gilad, Bresler et al. 2005; Liel, Shany et al. 1999; Schwartz, Smirnoff et al. 2000), liver (Duncan, Glass et al. 1991), and uterus (Levy, Zuili et al. 1984; Walters 1981). Gilad et al. showed that the enhanced expression of VDR by 17 β -estradiol is not through classical estrogen response elements, but through 1,25(OH)₂D binding to membrane-localized estrogen receptors that rapidly activate transcription of VDR through a Ras-Raf-MEK-ERK-cJUN pathway (Gilad, Bresler et al. 2005). However, not all cell types respond the same, as 17 β -estradiol inhibits VDR expression in the kidney (Duncan, Glass et al. 1991). Estrogens can also increase 1,25(OH)₂D concentrations locally by increasing CYP27B1 and decreasing CYP24 levels (Lechner, Bajna et al. 2006). In fact, in women shortly after the estrogen surge of the menstrual cycle, 1,25(OH)₂D serum levels are reportedly double what they are on day one of the menstrual cycle (Gray, McAdoo et al. 1982). In addition, progesterone has been reported to enhance estrogen-induced CYP27B1 expression (Tanaka, Castillo et al. 1978). Thus it appears that in general, vitamin D signaling is up-regulated by ovarian hormones. This is interesting because musculoskeletal pain is primarily associated with low estrogen status. For instance, a major side-effect of aromatase inhibitors, which prevent the production of estrogens, is musculoskeletal pain (Felson and Cummings 2005; Garreau, Delamelena et al. 2006; Morales, Pans et al. 2006; Mouridsen 2006). In addition, Leuprolide, a gonadotropin-releasing hormone agonist, which causes hypoestrogenemia, induces musculoskeletal pain in 25% of women who use it (Felson and Cummings 2005; Friedman, Juneau-Norcross et al. 1993). Furthermore, the prevalence for chronic widespread pain peaks in the 50's and 60's, corresponding with the age of menopause onset, a time when hormone levels are naturally dropping. In fact, it is well known that generalized musculoskeletal pain is a symptom of menopause (Dugan, Powell et al. 2006; Greendale, Reboussin et al. 1998). Asian women, who tend to have particularly low

estrogen levels during menopause (Cauley, Gutai et al. 1989; Randolph, Sowers et al. 2004) and are especially susceptible to vitamin D deficiency (Ford, Graham et al. 2006; Pal, Marshall et al. 2003; Shaw and Pal 2002), appear to be more susceptible to menopausal musculoskeletal pain, with one study reporting it as their most common menopausal symptom (Ho, Chan et al. 1999). Hormone replacement therapy, which is known to increase serum 1,25(OH)₂D₃ levels (van Hoof, van der Mooren et al. 1994; van Hoof, van der Mooren et al. 1999), has been shown to relieve and prevent the generalized pain associated with menopause (Dugan, Powell et al. 2006). In this study we examine whether the loss of ovarian hormones causes specific changes in the expression of vitamin D-related proteins in neurons that are part of the pain pathway.

The Need for an Animal Model

While clinical links between chronic musculoskeletal pain and vitamin D deficiency exist, confounding factors in humans make vitamin D deficiency difficult to analyze in isolation. Factors that contribute to a vitamin D deficiency, such as sex, skin color, smoking, body weight, socioeconomic status, age, and physical activity are also associated with musculoskeletal pain (Table 1). These factors may enhance susceptibility to vitamin D deficiency, which in turn cause musculoskeletal pain. Alternatively, vitamin D deficiency may be more likely to occur in individuals already susceptible to chronic musculoskeletal pain. An animal model of hypovitaminosis D-induced musculoskeletal pain will allow analysis without confounding factors. The studies described here will use a rat model, because rats are extensively used to study pain-related behavior and have been used to assess features of vitamin D deficiency for nearly 90 years (McCollum, Simmonds et al. 1922). Female rats will be used, because of the high prevalence of vitamin D deficiency and musculoskeletal pain in women (Andersson, Ejlertsson et al. 1993; Bergman, Herrstrom et al. 2001; Bolland, Grey et al. 2007; Dawson-Hughes, Harris et al. 1997; Rollman and Lautenbacher 2001).

Pain Associated with Hyperinnervation of Tissue

Increased peripheral nerve density is associated with several painful conditions (Di Sebastiano, Fink et al. 1995; Di Sebastiano, Fink et al. 1997; Fink, Di Sebastiano et al. 1994; Pang, Marchand et al. 1995; Reinert, Kaske et al. 1998; Reynolds and Fitzgerald 1995; Schubert, Weidler et al. 2005; Shinoda, Honda et al. 2003), and is known to occur in muscles, joints, and tendons (Reinert, Kaske et al. 1998; Schubert, Weidler et al. 2005; Shinoda, Honda et al. 2003). Steroid hormones other than vitamin D are known to regulate axonal sprouting, leading us to speculate whether vitamin D metabolites also had this potential (Blacklock, Johnson et al. 2005). Preliminary data were obtained, revealing that muscles from rachitic rats (a severe vitamin D deficiency model) had increased levels of the c-fiber specific intermediate filament protein, peripherin, suggesting that the muscles from vitamin D deficient might be hyperinnervated (Figure 1). Therefore, the focus of these studies was on how a vitamin D deficiency affects nociceptors, the first order neurons of the pain and temperature pathway, whose distal axons innervate peripheral tissue. In the first study described here, putative nociceptors were examined to determine whether they contain the machinery needed to metabolize and directly respond to vitamin D metabolites. In the second study, vitamin D status in rats was altered in order to determine whether a vitamin D deficiency can result in deep tissue hypersensitivity and muscle hyperinnervation.

TABLE

	Vitamin D levels	Musculoskeletal Pain
Sex	Vitamin D deficiency is more prevalent in women (Bolland, Grey et al. 2007; Dawson-Hughes, Harris et al. 1997).	Chronic widespread pain is more prevalent in women (Andersson, Ejlertsson et al. 1993; Bergman, Herrstrom et al. 2001; Rollman and Lautenbacher 2001).
Skin color	Due to the competition of melanin for UVB rays, synthesis of vitamin D in the skin is less efficient as skin pigmentation increases (Armas, Dowell et al. 2007; Clemens, Adams et al. 1982).	Chronic widespread pain prevalence is increased in African American women compared to Caucasian women (Gansky and Plesh 2007). In addition, black chronic pain patients experience a higher severity of pain (Edwards, Doleys et al. 2001; McCracken, Matthews et al. 2001).
Smoking	Smokers have a higher prevalence of vitamin D deficiency (Sowers, Wallace et al. 1986)	Smokers have a higher prevalence of musculoskeletal pain (Andersson, Ejlertsson et al. 1998; Brage and Bjerkedal 1996; Palmer, Syddall et al. 2003; Yunus, Arslan et al. 2002).
Body Weight	There is a negative correlation of vitamin D levels and body mass index (Arunabh, Pollack et al. 2003; Bischof, Heinze et al. 2006; Bolland, Grey et al. 2007; Need, Morris et al. 1993; Wortsman, Matsuoka et al. 2000).	There is a positive correlation between body mass index and musculoskeletal pain (Salaffi, De Angelis et al. 2005; Sievert and Goode-Null 2005; Yunus, Arslan et al. 2002).
Physical Activity	Exercise improves vitamin D activity (Bell, Godsen et al. 1988; Klausen, Breum et al. 1993; Nelson, Meredith et al. 1988).	Exercise alleviates chronic widespread pain (Jones, Adams et al. 2006; Linton and van Tulder 2001)
Age	Serum 25(OH)D levels decrease with age (Holick, Matsuoka et al. 1989; Need, Morris et al. 1993; Sowers, Wallace et al. 1986).	The prevalence of musculoskeletal pain increases with age, peaking between the ages of 55-65 and then decreasing slightly thereafter (Andersson, Ejlertsson et al. 1993; Croft, Rigby et al. 1993; Hunt, Silman et al. 1999; White, Speechley et al. 1999; Wolfe, Ross et al. 1995).

Table 1. Factors that contribute to both musculoskeletal pain and vitamin D deficiency.

FIGURE

Figure 1.

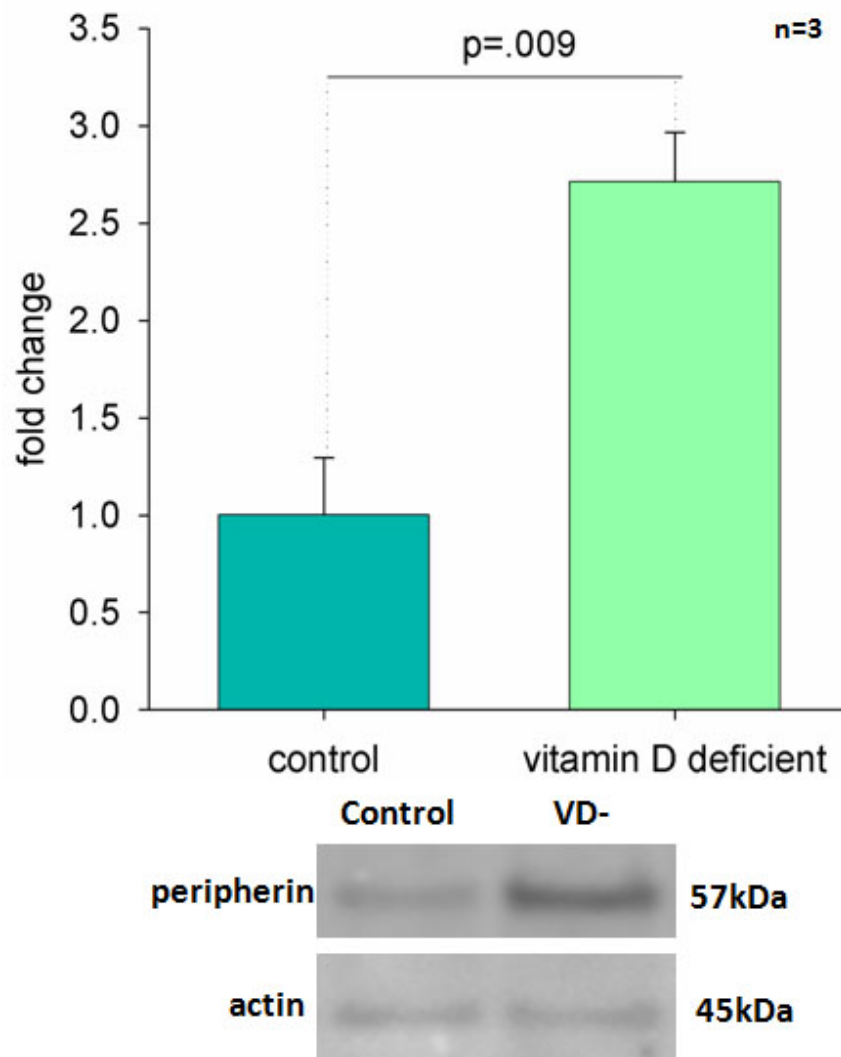


Figure 1. Increased peripherin content in muscles from vitamin D deficient rats. Western blots of lysates prepared from the lateral gastrocnemius muscles of control or rachitic vitamin D deficient rats. β actin was used to normalize intensity values, which are expressed in graphical form as fold change compared to control.

**CHAPTER 2. VITAMIN D RECEPTOR AND ENZYME EXPRESSION IN DORSAL ROOT
GANGLIA OF ADULT FEMALE RATS: MODULATION BY OVARIAN HORMONES**

ABSTRACT

Vitamin D insufficiency impacts sensory processes including pain and proprioception, but little is known regarding vitamin D signaling in adult sensory neurons. We analyzed female rat dorsal root ganglia (DRG) for vitamin receptor (VDR) and the vitamin D metabolizing enzymes CYP27B1 and CYP24. Western blots and immunofluorescence revealed the presence of these proteins in sensory neurons. Nuclear VDR immunoreactivity was present within nearly all neurons, while cytoplasmic VDR was found preferentially in unmyelinated calcitonin gene-related peptide (CGRP)-positive neurons, colocalizing with CYP27B1 and CYP24. These data suggest that 1,25(OH)₂D may affect sensory neurons through nuclear or extranuclear signaling pathways. In addition, local vitamin D metabolite concentrations in unmyelinated sensory neurons may be controlled through expression of CYP27B1 and CYP24. Because vitamin D deficiency appears to exacerbate some peri-menopausal pain syndromes, we assessed the effect of ovariectomy on vitamin D-related proteins. Two weeks following ovariectomy, total VDR expression in DRG dropped significantly, owing to a slight decrease in the percentage of total neurons expressing nuclear VDR and a large drop in unmyelinated CGRP-positive neurons expressing cytoplasmic VDR. Total CYP27B1 expression dropped significantly, predominantly due to decreased expression within unmyelinated CGRP-positive neurons. CYP24 expression remained unchanged. Therefore, unmyelinated CGRP-positive neurons appear to have a distinct vitamin D phenotype with hormonally-regulated ligand and receptor levels. These findings imply that vitamin D signaling may play a specialized role in a neural cell population that is primarily nociceptive.

INTRODUCTION

Vitamin D metabolites have widespread roles in human health. In addition to well known actions on calcium homeostasis and bone remodeling, vitamin D has been linked to immunity, cardiovascular disease, and cancer (Holick and Chen 2008). Accumulating evidence supports a role for vitamin D in certain types of pain. For example, vitamin D deficiency is associated with chronic musculoskeletal pain and acroparaesthesia (numbness, burning, or tingling in the extremities) (Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991; Masood, Narang et al. 1989; Plotnikoff and Quigley 2003). Over 85% of patients who report chronic musculoskeletal pain have levels of serum 25-hydroxyvitamin D₃ (25OHD₃) that are less than 20ng/ml (50nmol/L) (Benson, Wilson et al. 2006; Macfarlane, Palmer et al. 2005; Plotnikoff and Quigley 2003), which is relieved by vitamin D supplementation (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991). However, the mechanism by which vitamin D deficiency leads to altered sensation is unknown.

Vitamin D is derived from diet or exposure to sunlight, converted in the liver to the main circulating form, 25-hydroxyvitamin D (25OHD), which is further transformed by 25-hydroxyvitamin D 1 α -hydroxylase/cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1) into 1,25-dihydroxyvitamin D (1,25(OH)₂D), which is the most well understood active metabolite. Conversion of 25(OH)D into 1,25(OH)₂D occurs largely in kidney, but other cell types express CYP27B1 and can locally convert circulating precursor to active hormone (Jones 2007). 1,25(OH)₂D binds to vitamin D receptors (VDRs), which are spatially dynamic and may be localized within the nucleus where they influence gene transcription, or within the cytoplasm and membrane where they modulate ion flux and the generation of second messengers (Mizwicki and Norman 2009). Hydroxylation of 1,25(OH)₂D to form 1,24,25-trihydroxyvitamin D by the widely expressed enzyme, 25-hydroxyvitamin D 24-hydroxylase/cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24), is an initial step in the pathway of 1,25(OH)₂D degradation, but CYP24 also produces other vitamin D metabolites, whose actions are just beginning to be understood (Omdahl, Bobrovnikova et al. 2003; Sakaki, Kagawa et al.

2005). The extent to which these vitamin D-related proteins are present within adult sensory neurons is unknown.

A number of factors can influence VDR signaling, including ovarian hormones. For instance, VDR expression is generally up-regulated by 17 β -estradiol (Gilad, Bresler et al. 2005). Further, estrogens can increase 1,25(OH)₂D concentrations locally by increasing CYP27B1 and decreasing CYP24 levels (Lechner, Bajna et al. 2006). In addition, progesterone has been reported to enhance estrogen-induced CYP27B1 expression (Tanaka, Castillo et al. 1978). While it is not known whether ovarian hormones and vitamin D signaling mechanisms interact in sensory nerve pathways, peripheral sensory neurons do express estrogen and progesterone receptors (Chan, Rodriguez-Waitkus et al. 2000; Sohrabji, Miranda et al. 1994), and anecdotal evidence suggests that vitamin D and ovarian hormones may interact to influence pain sensation. For example, musculoskeletal pain is more prevalent in females and frequently exacerbated by menopause or pharmacological estrogen suppression (Andersson, Ejlertsson et al. 1993; Bergman, Herrstrom et al. 2001; Croft, Rigby et al. 1993; Dugan, Powell et al. 2006; Felson and Cummings 2005; Friedman, Juneau-Norcross et al. 1993; Garreau, Delamelena et al. 2006; Greendale, Reboussin et al. 1998; Morales, Pans et al. 2006; Mouridsen 2006; White, Speechley et al. 1999; Wolfe, Ross et al. 1995). Moreover, musculoskeletal pain induced by hypoestrogenemia is inversely correlated with serum 25(OH)D levels and can be partially ameliorated by high-dose vitamin D₃ therapy (Khan, Reddy et al. 2009; Waltman, Ott et al. 2009). To assess the potential for vitamin D signaling in peripheral sensory neurons and the possible influence of reproductive hormones, we examined expression patterns of VDR, CYP27B1, and CYP24 in adult dorsal root ganglia (DRG) in intact and ovariectomized female rats.

MATERIALS AND METHODS

Experimental preparations

Five female Sprague Dawley (Harlan Laboratories, Indianapolis, IN) rats at six weeks of age were ovariectomized by bilateral hind flank incision following anesthesia with 60mg/kg ketamine (Pfizer,

New York, New York), 0.4mg/kg atropine (Baxter, Deerfield, IL), 8mg/kg xylazine (Lloyd Laboratories, Shenandoah, Iowa). Four age-matched female rats were allowed to cycle normally and estrous cycle stage was tracked daily by vaginal lavage with an eyedropper and 0.9% saline (Becker, Arnold et al. 2005). After two weeks, all rats were euthanized by i.p. injection of sodium pentobarbital (150mg/kg, Ovation) followed by decapitation; cycling rats cycled normally during this two week period and were euthanized at estrus, as determined by an opaque vaginal smear consisting predominantly of cornified epithelial cells. All rats were exposed to a 14hr light/ 10hr dark cycle and sacrificed 4-6 hr into the light cycle. All procedures were reviewed by the University of Kansas Medical Center Institutional Animal Care and Use Committee and conformed to all local and federal guidelines.

DRGs from C3-S1 were removed and all left ganglia from each animal were pooled and stored in RNAlater (Ambion, Austin, Texas) at -20°C for western blot protein analysis. The right DRGs were embedded in tissue chilled freezing medium (Electron Microscopy Sciences, Hatfield, PA), snap frozen, and stored at -80°C for immunofluorescence studies.

Antibody Characterization

In preliminary experiments several anti-VDR antibodies were tested by immunohistochemistry on rat DRG sections. Antibodies GTX73019 (GeneTex, Irvine, CA), and Santa Cruz antibodies C-20, H81, and D-6 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) all had very similar staining patterns, we chose to use the C-20 antibody, because a blocking peptide was available to confirm specificity. The rabbit anti-VDR antibody C-20 was raised against a region within the last 50 amino acids on the C-terminal end of the rat VDR protein. The antibody recognized a band of the expected size (50-55kDa) as well as several bands that have previously been shown with this antibody and other VDR antibodies (Gonzalez Pardo, Boland et al. 2008; Nangia, Butcher et al. 1998; Wang, Becklund et al. 2010). The sheep anti-CYP27B1 (The Binding Site, Birmingham, UK) was raised against a murine CYP27B1 peptide (RHVELREGAAMRNQGKPEEDMPS) and recognized two bands, corresponding to sizes expected for full length CYP27B1 (56kDa) and a known splice variant that has no enzymatic activity (25kDa) (Diesel,

Radermacher et al. 2005). The goat anti-CYP24 G-15 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Ca) was raised against 15 amino acids between amino acids 400-450 of the human CYP24a protein and recognized a single band corresponding to the expected size (55kDa). All western blot bands and immunofluorescent staining with the above antibodies was almost completely abolished after overnight preincubation of the primary antibodies with the following blocking peptides (1:5 antibody:blocking peptide): VDR C-20 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca), CYP27B1 G-20 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca), CYP27B1 G-15 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca).

Peripherin selectively identifies unmyelinated neurons (Goldstein, House et al. 1991). Because of potential interactions with double staining, two different peripherin antibodies were used. Chicken IgY anti-peripherin AB9282 (Millipore, Billerica, MA) was raised against recombinant peripherin protein and rabbit anti-peripherin AB1530 (Millipore, Billerica, MA) was raised against a trp-E fusion protein containing all but the four N-terminal amino acids. The rabbit anti-peripherin antibody has been previously characterized (Tseng, Chau et al. 2008), and the percentage of DRG neurons expressing peripherin was similar to other accounts (Table 1) (Goldstein, House et al. 1991). Double staining of DRG with both the chicken and rabbit antibodies revealed identical staining patterns.

Calcitonin gene-related peptide (CGRP) resides within NGF-dependent sensory neurons (Lawson 1992), and again two separate antibodies were used. Goat anti-CGRP P01256 (AbD Serotec, Raleigh, NC) was raised against synthetic rat CGRP conjugated to gamma globulin, and sheep anti-CGRP CA1137 (Enzo Life Sciences, Plymouth Meeting, PA) was raised against synthetic rat CGRP conjugated to bovine serum albumin. These two antibodies had identical staining patterns, which have been previously characterized (Ruscheweyh, Forsthuber et al. 2007; Yasuhara, Aimi et al. 2008), and the percentage of DRG neurons expressing CGRP was similar to that reported by others (Table 1) (Lawson 1992).

Western Blots

Pooled left DRGs were homogenized in 350 μ l of RP1 buffer (Machery-Nagel, Düren, Germany) with 3.5 μ l of β -mercaptoethanol (Sigma-Aldrich Corp, St. Louis, MO) and proteins extracted using a

Protein/RNA Nucleospin kit (Machery-Nagel, Düren, Germany). Proteins were separated side by side on 4-12% Bis-Tris precast gels (Invitrogen, Carlsbad, Ca) and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk, 0.1% bovine serum albumin, and 2% normal serum from the secondary antibody host. Blots were probed overnight at 4°C using anti-VDR C-20 (1:50 rabbit IgG, Santa Cruz), anti-CYP27B1 (1:50 sheep IgG, The Binding Site), or anti-CYP24 G-15 (1:50 goat IgG, Santa Cruz). Secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase were added for 2hr at room temperature. Blots were developed with either Biorad Immuno-Star AP substrate (Bio-Rad, Hercules, CA) or Thermo Scientific SuperSignal Chemiluminescent substrate (Rockford, IL). Blots were stripped (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL) and reprobed with 1:2000 mouse anti-GAPDH (Millipore, Billerica, MA). Images were acquired on a Molecular Imager ChemiDoc XRS system (Bio-Rad, Hercules, CA) and analyzed with Quantity One software (Bio-Rad, Hercules, CA). Adjusted volumes ($\text{Intensity} \cdot \text{mm}^2$) of bands of interest were normalized to GAPDH in the same lane, and values for animal replicates averaged. Significance ($p \leq 0.05$) was determined by student's t-test. The images presented here have been cropped to show representative lanes/bands and adjusted for brightness and contrast.

Immunofluorescence

DRGs from each animal were sectioned at a thickness of 10 μm and stored at -80°C. Thawed sections were post-fixed 30 min in 4% formaldehyde freshly-prepared from paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS). Slides were incubated 20 min in blocker containing 30% normal serum (Equitech-Bio Inc., Kerrville, TX) from the secondary antibody host, 0.5% triton X-100 (Sigma-Aldrich, St. Louis, MO), 0.54% ammonium chloride (Sigma-Aldrich, St. Louis, MO), and 0.07% porcine gelatin (Sigma-Aldrich, St. Louis, MO). Sections were incubated overnight at room temperature with primary antibody at the following dilutions: 1:50 rabbit anti-VDR C-20, 1:25 Sheep anti-CYP27B1, 1:25 goat anti-CYP24 G-15, 1:1000 chicken IgY (or 1:400 rabbit anti-peripherin, 1:200 goat or Sheep (Biomol) anti-CGRP. Isolectin IB4 (IB4) conjugated to Alexa Fluor 488

(Invitrogen, Carlsbad, Ca), which identifies unmyelinated sensory neurons that are responsive to the GDNF family of ligands (Ambalavanar and Morris 1993; Bennett, Michael et al. 1998), was added to slides for 20min between the primary and secondary antibodies at a concentration of 2µg/ml in PBS containing 1mM CaCl₂ (Fisher Scientific, Pittsburgh, PA). Nuclei were stained with 400 nM 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) for 10 min before the addition of secondary antibodies. Secondary antibody incubations were conducted at room temperature for 2 hrs at the following concentrations: 1:200 goat anti-chicken IgY Cy2, 1:500 donkey anti-rabbit Alex Flour 647 (Invitrogen, Carlsbad, CA), 1:200 donkey anti-sheep Cy3 (Jackson ImmunoResearch, West Grove, PA), 1:1000 donkey anti-sheep Alexa Fluor 488 (Invitrogen, Carlsbad, CA), or 1:200 donkey anti-goat Cy3 (Jackson ImmunoResearch, West Grove, PA). All antibodies were diluted in PBS containing 23% donkey serum, and slides were rinsed between staining steps. No staining was observed when primary antibodies were omitted and staining was almost completely abolished after preincubation of the primary antibodies with their blocking peptides.

Neuronal Quantitation

We quantitatively assessed immunoreactivity (ir) of vitamin D-related proteins and neural markers in the L5 DRG, because its relatively large size provides a substantial number of neurons to sample. Moreover, L5 DRG should have very few, if any, projections to the ovary, reproductive tract, or incision sites and therefore should not be damaged by the ovariectomy procedure (Berkley, Robbins et al. 1993; Steinman, Carlton et al. 1992; Takahashi, Chiba et al. 2003; Tanaka, Matsugami et al. 2002). (No gross differences were observed between staining in L5 and the other vertebral levels of DRG for any of the proteins examined.) For each stain, sections 250µm apart throughout each ganglion were imaged on a Nikon 80i fluorescent microscope. A standard threshold value was visually determined for each stain, which appeared to differentiate real staining from background fluorescence. This standard threshold value was used to create a mask for each image so that neurons with clear DAPI-stained nuclei and staining intensities above the standard threshold could be manually counted without bias using

Metamorph software. Counting sections 250 μ m apart throughout the ganglion (3-5 sections) yielded an average of 428 \pm 42 total neurons with clearly stained nuclei for each DRG. We further assessed cell types in which vitamin D-associated proteins reside by co-immunostaining for peripherin, CGRP, or IB4 and using the same color thresholding techniques for merged images as described above. We also characterized the intracellular localization of VDR to determine if; 1) Intensely stained VDR-immunoreactive aggregates were present within the DAPI-stained nuclear area, or 2) VDR-immunoreactive-staining above the standard threshold was found outside the DAPI-stained nuclear area for each neuron. For each animal and stain, percentages of labeled neurons were computed from averages of all sections analyzed, and data are presented as the mean \pm standard error. Significance ($P \leq 0.05$) was determined by ANOVA followed by post-hoc analysis using Student-Newman-Keuls (SNK) or student's t-test. For publication images were pseudocolored and adjusted for brightness and contrast.

RESULTS

Vitamin D-related proteins in dorsal root ganglia of rats at estrus

Vitamin D receptor

We assessed DRGs from intact female rats in estrus for the presence of VDRs. Western blots of total DRG protein revealed a strong band at approximately 60kDa. Two fainter bands were also consistently observed at 41kDa, and 24kDa and three very faint bands at 74kDa, 65kDa, and 50kDa could also be identified (Fig. 1). Immunofluorescence of sectioned DRG revealed VDR-ir in neurons, but no VDR-ir surrounding the neurons or within central nerve bundles where satellite glia and Schwann cells are located (Fig. 2A). Most neurons displayed VDR-ir localized to small nuclear aggregates; however, some neurons also had diffuse staining throughout the cell body or patchy membrane-localized staining. For this study we will refer to diffuse VDR-ir as cytoplasmic, although some of this staining may be confined to vesicles dispersed throughout the cytoplasm.

The percentage of total neurons with nuclear (nVDR) or cytoplasmic (cVDR) VDR-ir was quantified in L5 DRG (Fig. 2G). About 71% of all neurons express nVDR, while 10% had cVDR staining and 8% had both nVDR and cVDR-ir.

To determine whether VDR is differentially expressed by small or large fiber neurons, DRG sections were co-immunostained for VDR and the small fiber specific intermediate filament peripherin (Fig. 3A). Peripherin-positive and peripherin-negative neurons with clear DAPI stained nuclei were counted (DAPI-staining is not shown). In intact rats, the percentage of neurons with nVDR did not differ between the peripherin-negative and peripherin-positive neuronal subpopulations (Fig. 3G). On the other hand, cVDR was preferentially expressed by small fiber peripherin-positive neurons (Fig. 3H). The percentage of peripherin-ir neurons expressing cVDR was more than twice that of peripherin-negative neurons ($p=0.002$).

To determine whether VDR is differentially expressed by a specific subset of small fiber neurons, DRG sections were co-labeled for VDR and IB4 (Fig. 3B), which stains small fiber neurons responsive to the GDNF family of ligands, or CGRP (Fig. 3C), which is found in NGF-dependent neurons. nVDR expression did not differ between the two neuronal sub-types (Fig. 3I), while the percentage of CGRP neurons that expressed cVDR was almost three times that of IB4-positive neurons (Fig. 3J; $p=0.016$).

CYP27B1

We assessed whether DRG from intact female rats in estrus express CYP27B1, the enzyme that converts circulating 25(OH)D into active 1,25(OH)₂D. Western blots revealed two bands that migrated at approximately 56kDa and 25kDa (Fig. 1).

Immunofluorescent-staining of sectioned DRG revealed CYP27B1-ir in DRG neurons but not glia (Fig. 2B). CYP27B1-ir was diffuse throughout neural cell bodies. The percentage of total neurons expressing CYP27B1 was about 22% (Fig. 2H).

DAPI-stained DRG sections were immunofluorescently-labeled for CYP27B1 and peripherin to determine whether CYP27B1 is differentially expressed by small or large fiber neurons (Fig. 4A, DAPI-

staining is not shown). The percentage of peripherin-ir neurons expressing CYP27B1 was more than three times that of peripherin-negative neurons (Fig 4G; $p < 0.001$).

DRG sections were immunofluorescently-labeled for CYP27B1 and IB4 (Fig. 4B) or CGRP (Fig. 4C) to determine whether expression occurs in specific neuronal subsets. The percentage of CGRP-ir neurons that expressed CYP27B1 was more than two times the percentage of IB4-positive expressing CYP27B1 (Fig. 4H; $p = 0.003$).

CYP24

Western Blot was used to determine whether DRG from intact female rats in estrus express CYP24, an enzyme involved in the degradation of $1,25(\text{OH})_2\text{D}$ and the production of other vitamin D metabolites. A single faint band of the expected size of 55kDa was identified on western blots run with total DRG protein (Fig. 1).

Immunofluorescence of sectioned DRG revealed CYP24-ir in DRG neurons but not glia (Fig. 2C). Like CYP27B1-ir, CYP24-ir was diffuse throughout neural cell bodies. The percentage of total neurons with CYP24 was quantified in the L5 DRG of intact female rats in estrus. About 17% of all neurons express CYP24 (Fig. 2I).

To determine whether CYP24 is differentially expressed by large or small fiber neuronal cell types, DRG sections were co-labeled for CYP24 and peripherin (Fig. 5A). CYP24 was preferentially expressed by small fiber peripherin-positive neurons (Fig. 5G). The percentage of peripherin-ir neurons expressing CYP24 was one and a half times that of peripherin-negative neurons.

We assessed the extent to which CYP24 is expressed by peptidergic or non-peptidergic small fiber neurons. DRG sections were immunofluorescently-labeled for CYP24 and CGRP (Fig. 5C) or IB4 (Fig. 5B). The percentage of CGRP-positive neurons expressing CYP24 was about four times more than the percentage of IB4-positive neurons expressing CYP24 (Fig. 5H; $p < 0.001$).

cVDR colocalizes with CYP27B1 and CYP24

Because cVDR, CYP27B1, and CYP24 were all preferentially expressed by peripherin-ir, CGRP-ir neurons, we co-stained DRG for cVDR and CYP27B1 (Fig. 6A) or CYP24 (Fig. 6B) to determine if they are expressed within the same neurons. Most neurons with cVDR-ir were observed to have CYP27B-ir and CYP24-ir, showing that neurons with cVDR typically have proteins necessary to locally regulate 1,25(OH)₂D concentrations.

Effect of ovariectomy (OVX) on Vitamin D-related proteins in dorsal root ganglia

VDR

Having verified that DRG neurons from intact female rats express VDR, CYP27B1, and CYP24, we assessed whether expression of these proteins is affected by OVX. We began by examining whether OVX alters VDR protein levels in total DRG lysates (Fig. 7A). The only VDR isoform to change significantly following OVX was the 60kDa band, which was reduced by 51% compared to lysates from intact rats in estrus (p=0.037).

Immunohistochemistry revealed that, as in intact rats, VDR-ir in ovariectomized rats was distributed in nuclear and cytoplasmic compartments in neurons, but not glia (Fig. 2D). Approximately 66% of neurons in DRG from ovariectomized rats express nVDR, 8% express cVDR, and 6% express both (Fig. 2G); only the modest reduction in nVDR was significant (p=0.038).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for VDR and peripherin (Fig. 3D). The percentage of peripherin-ir and peripherin-negative DRG neurons expressing nVDR in ovariectomized rats did not change (Fig. 3G). However, the percentage of peripherin-ir neurons with cVDR was reduced, so that there was no longer any difference in the percentage of peripherin-ir and peripherin-negative neurons expressing cVDR in ovariectomized rats (Fig. 3H).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for VDR and IB4 (Fig. 3E) or CGRP (Fig. 3F). The percentage of DRG neurons expressing nVDR in ovariectomized rats was

comparable in CGRP-ir and IB4-positive neurons (Fig. 3I). However, there was a 63% decrease in the percentage of CGRP-ir expressing cVDR following OVX (Fig. 3J; $p=0.009$).

CYP27B1

Western blots of total DRG protein showed that while there was no change in expression of the 25kDa CYP27B1 band, expression of the 56kDa CYP27B1 band dropped by 31% following OVX (Fig. 7B; $p=0.042$).

CYP27B1-ir in DRG from ovariectomized rats was found in neurons, but not glia (Fig. 2E). The average percentage of total neurons expressing CYP27B1 dropped from $22\pm4\%$ to $10\pm2\%$ following OVX, but this change did not achieve statistical significance (Fig. 2H; $p=0.063$).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for peripherin and CYP27B1 (Fig. 4D). Ovariectomy did not affect the small proportion of peripherin-negative neurons expressing this enzyme. (Fig. 4G). However, OVX resulted in a 57% decrease in proportion of peripherin-ir neurons expressing CYP27B1 ($p=0.003$) to a level comparable to that of peripherin-negative neurons (Fig. 4G).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for CYP27B1 and IB4 (Fig. 4E) or CGRP (Fig. 4F). Ovariectomy did not significantly alter the proportion of IB4-positive neurons expressing CYP27B1 (Fig. 4H). In contrast, there was a 61% drop in the proportion of CGRP-ir DRG neurons with CYP27B1-ir after OVX (Fig. 4H; $p<0.001$), such that percentages of neurons expressing this enzyme were comparable in IB4 and CGRP-ir subpopulations.

CYP24

Western blots of total DRG protein showed that there was no significant change in total CYP24 levels following OVX (Fig. 7C). As in rats at estrus, CYP24-ir was present exclusively in neurons (Fig. 2F), and immunostaining showed no change in the percentage of total neurons expressing CYP24 following OVX (Fig. 2I).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for peripherin and CYP24 (Fig. 5D). As with rats in estrus, CYP24-ir was preferentially expressed by small fiber peripherin-positive neurons in OVX rats (Fig. 5G). Ovariectomy did not alter the proportion of peripherin-ir or peripherin negative neurons displaying CYP24-ir (Fig. 5G).

DAPI-stained DRG sections from OVX rats were co-labeled for CYP24 and IB4 (Fig. 5E) or CGRP (Fig. 5F). As in estrus, CYP24-ir was present predominantly within CGRP-ir neurons, with only small numbers of IB4-positive neurons expressing this enzyme (Fig. 5H). Ovariectomy did not alter numbers of either CGRP-ir or IB4-positive neurons expressing CYP24 (Fig. 5H).

Changes in neural markers following ovariectomy

We assessed whether changes in neuronal protein expression following OVX could contribute to observed differences in expression of vitamin D-related proteins. The percentage of total neurons expressing peripherin was 63% in intact rats and underwent a small but significant decline to 59% following OVX ($p=0.042$, Table 1). Within the peripherin-positive population of DRG neurons, OVX resulted in a 38% decline in the percentage of neurons expressing CGRP-ir, while the IB4-positive population was unaffected (Table 1).

The small decrease in peripherin-positive neurons resulted in a proportional increase in peripherin-negative neurons, but the small numbers of these neurons expressing CGRP or IB4 were unaffected (Table 1). The percentages of IB4-positive and CGRP-ir neurons that expressed peripherin-ir were unaltered by OVX (Tables 1).

DISCUSSION

The central finding of this study is that adult rat sensory neurons express proteins associated with vitamin D signaling pathways. Nuclear VDR is widely distributed within all types of DRG neurons suggesting broad transcriptional actions of this hormone. Membrane and cytoplasmic VDR, perhaps indicative of rapid signaling pathways, is present selectively in unmyelinated, CGRP-ir neurons. This

same subpopulation contains vitamin D metabolizing enzymes, implying close regulation of vitamin D metabolite levels. Ovarian hormones may play an important role in regulating neuronal responsiveness to $1,25(\text{OH})_2\text{D}$ by up-regulating both its activating enzyme and its cytoplasmic receptor. Because vitamin D deficiency is strongly associated with musculoskeletal pain and this is exacerbated by the loss of ovarian hormones, it is important to consider the role of these convergent systems in regulating nociceptor function.

Vitamin D signaling in the central nervous system

Vitamin D is becoming recognized as an important hormone in nervous system health. VDR is expressed by neurons, oligodendrocytes, and astrocytes within the limbic system, basal ganglia, spinal cord, cerebellum, and cerebral cortex (Baas, Prufer et al. 2000; Eyles, Smith et al. 2005; Glaser, Veenstra et al. 1999; Neveu, Naveilhan et al. 1994; Perez-Fernandez, Alonso et al. 1997; Prufer, Veenstra et al. 1999; Walbert, Jirikowski et al. 2001). VDR localization within both neuronal nucleus and cytoplasm has been described (Glaser, Veenstra et al. 1999; Prufer, Veenstra et al. 1999; Racz and Barsony 1999). Studies have also shown that neurons and glia in brain express CYP27B1 (Eyles, Smith et al. 2005; Zehnder, Bland et al. 2001), and CYP24 mRNA has been detected in glioma and primary glial cell culture (Naveilhan, Neveu et al. 1993), suggesting that the brain can regulate local $1,25(\text{OH})_2\text{D}$ concentrations. Vitamin D signaling has been linked to cell survival, proliferation, and differentiation, and is especially important during brain development (Levenson and Figueiroa 2008). In fact, gestational vitamin D deficiency is thought to be associated with long term cognitive disorders such as anxiety and schizophrenia (Levenson and Figueiroa 2008). In the adult, vitamin D supplementation has been proposed to improve outcomes in multiple sclerosis, Parkinson's disease, and traumatic brain injury (Cekic, Sayeed et al. 2009; Myhr 2009; Newmark and Newmark 2007). Thus, accumulating evidence suggests that vitamin D signaling plays important roles in the central nervous system.

Vitamin D-related proteins in adult rat DRG

Much less is known regarding the role of vitamin D in the peripheral nervous system. VDR has been identified in fetal (E12-E21) DRG neurons but not glia (Johnson, Grande et al. 1996; Veenstra, Prufer et al. 1998) (although VDR mRNA was reported in cultured sciatic nerve Schwann cells (Cornet, Baudet et al. 1998)). However, the presence of VDRs and related proteins in the adult peripheral nervous system is not well documented.

Our western blot findings show that VDR protein is present within adult rat DRG, and immunocytochemical analyses show that it is found in most neurons. VDR can regulate transcription by forming homodimers or heterodimers with 9-cis retinoid X receptor (RXR) which then translocate to the nucleus (Shaffer and Gewirth 2004). Consistent with a transcriptional role, nuclear VDR was observed in the majority of DRG neurons as small discrete foci (Glaser, Veenstra et al. 1999; Prufer, Veenstra et al. 1999); because these may be absent from a given section plane of a given neuronal nucleus, we may have underestimated their actual prevalence. These foci are believed to represent binding of ligand-activated RXR/VDR heterodimers to DNA, thus corresponding to sites of transcription (Prufer, Racz et al. 2000). Because nuclear VDR was distributed widely throughout all DRG neural subtypes, VDR is likely to act as a classical transcription factor in most adult DRG neurons.

We also observed a subset of neurons where VDR-ir was localized to the cytoplasm or plasma membrane. While some of the cytosolic VDR could represent *de novo* synthesis or ligand-deficient VDR dimers (Prufer, Racz et al. 2000; Racz and Barsony 1999), another explanation is that this VDR fraction is involved in rapid signaling. The most prominent band disclosed in DRG homogenates had a mass of 60kDa, and this isoform reportedly is exclusive to membrane and cytosolic subcellular fractions (Gonzalez Pardo, Boland et al. 2008). In cardiomyocytes, cVDR serves as a reserve which, upon 1,25(OH)₂D binding, translocates to cardiac t-tubules (Tishkoff, Nibbelink et al. 2008). The patchy VDR-ir pattern localized to the plasma membrane of some DRG neurons suggests a distribution to distinct membrane microdomains, and is consistent with a role for VDR in rapid signaling in sensory neurons (Huhtakangas, Olivera et al. 2004). For example, 1,25(OH)₂D can influence calcium channel activity,

leading to rapid increases in intracellular calcium concentration and production of second messengers (Losel, Falkenstein et al. 2003). Thus, in addition to its transcriptional role within all DRG neuronal populations, VDR may exert rapid signaling in a selected subpopulation of neurons.

VDR signaling in DRG neuronal subpopulations

Selective neuronal markers provide insight as to how VDR signaling may vary as a function of neuronal subtype. Neurons lacking the intermediate filament protein peripherin are predominantly large myelinated neurons (Goldstein, House et al. 1991), which are involved in tactile sensation and proprioception (Casellini and Vinik 2007; Muller, Ryals et al. 2008; Nardone, Galante et al. 2007). These neurons contained nVDR but were mostly devoid of both CYP27B1-ir and CYP24-ir. This implies that large sensory neurons may be influenced by VDR transcriptional signaling, but that VDR activation is determined passively by levels of circulating 1,25(OH)₂D, as they likely lack the ability to regulate local 1,25(OH)₂D levels. The functional significance of this signaling pathway is unclear, but it is noteworthy that vitamin D deficiency in humans is associated with increased incidence in falls (Bischoff-Ferrari, Dawson-Hughes et al. 2004; Faulkner, Cauley et al. 2006), which may suggest altered proprioceptive function of large sensory fibers.

Neurons containing peripherin-ir represent unmyelinated C-fibers, which are associated with chemoreception, thermal sensation, and pain, burning and itching. As with large myelinated neurons, the majority of these also showed VDR-ir localized to the nucleus. However, these peripherin-ir neurons also include the subgroup in which VDR is localized to the cytoplasm, most often in conjunction with nuclear staining. It is well known that C-fiber neurons can be further characterized based on their neurotrophin dependencies and peptide content, and our colocalization studies show that the cytoplasmic form of VDR is distributed selectively in the NGF-dependent, CGRP-ir neurons, with approximately one third displaying cVDR; these neurons conduct information regarding pain and temperature, and antidromically release neuropeptides that promote vasodilation and extravasation, thus contributing to inflammation

(Birklein and Schmelz 2008). These findings suggest that vitamin D can activate multiple signaling pathways, which may functionally influence a subpopulation of neurons with pain-sensing capabilities.

Our observations regarding VDR distribution in the DRG suggest that some neurons may be more strongly influenced by vitamin D than others. If so, then local vitamin D metabolite levels for those neurons may require closer regulation. Findings concerning vitamin D-related proteins provide evidence that this is the case. Hence, the prohormone converting enzyme CYP27B1 is enriched specifically in peripherin-ir, CGRP-ir neurons, as is CYP24, which could be involved in the inactivation of $1,25(\text{OH})_2\text{D}$ or perhaps the formation of other metabolites such as $24,25(\text{OH})_2\text{D}_3$. Further, these enzymes co-localize selectively within neurons displaying cytoplasmic VDR. Collectively, these findings are consistent with the hypothesis that DRG peptidergic neurons possess vitamin D signaling pathways, whose gain can be modulated by altering levels of cVDR, or by regulating levels of vitamin D metabolic enzymes.

Regulation of VDR and CYP27B1 by ovarian hormones

One likely factor in regulating vitamin D-related proteins in DRG is female reproductive hormone status. Ovarian hormones have been found to promote the formation of $1,25(\text{OH})_2\text{D}$ by enhancing CYP27B1 expression and by inhibiting the expression of CYP24 (Lechner, Bajna et al. 2006; Tanaka, Castillo et al. 1978). They are also reported to promote the expression of VDR (Gilad, Bresler et al. 2005). In cancer models there has been some progress in uncovering how estradiol regulates VDR expression. Estradiol binds membrane bound $\text{ER}\beta$ receptors, which signal through ERK1/2 to increase transcription of VDR (Gilad, Bresler et al. 2005). Research from another group has shown that estrogen may regulate VDR promoter activity of exon 1C through SP1 sites, which can be mediated through $\text{ER}\alpha$ or $\text{ER}\beta$ (Wietzke, Ward et al. 2005). To determine whether ovarian hormones regulate VDR or enzyme expression in DRG neurons, we compared vitamin D-related protein expression in DRG from cycling rats at estrus to that of ovariectomized rats. Estrus occurs several hours after the natural surge in reproductive hormones including estradiol, progesterone, and prolactin (Freeman 1994), when hormone-responsive changes in protein expression are most apt to be evident. In contrast, rats following OVX have sustained

depression of ovarian hormones similar to that which occurs naturally in menopause, albeit occurring at a younger age and more abruptly than would occur normally.

Consistent with reported findings for other tissues, OVX resulted in decreased expression of VDR and CYP27B1. In protein blots, the cytoplasm/membrane-specific 60kDa isoform of VDR was reduced by 51%, and the 56kDa full length CYP27B1 band was reduced by 31%. Immunofluorescence examination showed that the numbers of neurons with cVDR and CYP27B1 expression were both diminished, and this decrease occurred exclusively within the peripherin-ir, CGRP-ir population. In contrast, no significant changes occurred in nVDR or CYP24. Therefore, based on these findings in OVX rats, a decline in ovarian hormones would not only down-regulate cVDR expression, but would also decrease the amount of CYP27B1 available to convert prohormone to active 1,25(OH)₂D. At the same time, levels of the CYP24 remain unchanged, thereby favoring a reduction in levels of 1,25(OH)₂D. Accordingly, low ovarian hormone levels may impair vitamin D signaling in peptidergic nociceptor neurons by diminishing levels of both cytoplasmic receptor and ligand.

While these observations are consistent with a reduction in the numbers of neurons in which cVDR signaling occurs, a caveat is that changes in reproductive hormones could influence marker expression, thereby introducing error to our counts of neuronal subpopulations containing vitamin D-related proteins. While hormone-related changes in IB4-positive neurons have not been reported (and were not detected here), it is known that estrogens increase CGRP expression (Gangula, Lanlua et al. 2000; Mowa, Usip et al. 2003). Consistent with these reports, we found that OVX resulted in a reduction of roughly 1/3 in the number of neurons expressing CGRP-ir. Of those retaining the CGRP phenotype, cVDR and activating enzyme expression were reduced by over 60%. This occurred without concomitant increases in cVDR or CYP27B1 in other cell phenotypes that might imply changes due to altered marker expression. It therefore appears that ovarian hormone deficiency selectively affects a subpopulation of DRG neurons, where both peptide phenotype and VDR signaling are dramatically reduced.

Vitamin D, ovarian hormones, and pain

While an association between pain and vitamin D deficiency has been observed for at least 20 years (Masood, Narang et al. 1989), this is often attributed to bone and muscle pathologies. Muscle symptoms, including pain, precede the appearance of biochemical indicators of bone pathology (Glerup, Mikkelsen et al. 2000; Masood, Narang et al. 1989), suggesting that bone is not the only source of musculoskeletal pain. Vitamin D deficiency does lead to atrophy of type II muscle fibers (Glerup, Mikkelsen et al. 2000); however, type II muscle fiber atrophy is non-specific and does not in itself cause pain (Mastaglia and Hilton-Jones 2007). Based on our findings, it seems likely that vitamin D may act directly on sensory nerves to modulate pain. Moreover, the extent to which vitamin D deficiency produces musculoskeletal pain appears to be strongly linked to estrogen status. For example, musculoskeletal pain occurs commonly in hypoestrogenemia resulting from naturally occurring menopause (Andersson, Ejlertsson et al. 1993; Bergman, Herrstrom et al. 2001; Croft, Rigby et al. 1993; Dugan, Powell et al. 2006; Greendale, Reboussin et al. 1998; White, Speechley et al. 1999; Wolfe, Ross et al. 1995) or pharmacotherapy using aromatase inhibitors or gonadotropin-releasing hormone agonists (Felson and Cummings 2005; Friedman, Juneau-Norcross et al. 1993; Garreau, Delamelena et al. 2006; Morales, Pans et al. 2006; Mouridsen 2006). Musculoskeletal pain associated with menopause is ameliorated by hormone replacement therapy (Dugan, Powell et al. 2006), implying a direct link between pain and hormone levels. It is not clear why musculoskeletal pain occurs with hypoestrogenemia. However, low estrogen levels following aromatase inhibition are accompanied by low serum 25(OH)D levels, and high dose vitamin D₃ supplementation partially reverses the discomfort (Khan, Reddy et al. 2009; Waltman, Ott et al. 2009), consistent with a link among estrogens, vitamin D and pain. However, it should be noted that high dose vitamin D₃ treatment does not fully alleviate pain symptoms. This may not be surprising given our findings that low ovarian hormone levels are also associated with diminished DRG neuronal expression of VDR and CYP27B1. Thus, hypoestrogenemia might hinder conversion of the therapeutic supplement to the active hormone and limit receptor-mediated signaling, thereby rendering dietary supplementation less effective. Collectively, these studies and the present findings

underscore a need for additional research clarifying the relationships among vitamin D, steroid hormones, and sensory pathways.

TABLES

	Total neurons %	Peripherin- positive neurons %	Peripherin- negative neurons %	IB4 neurons %	CGRP neurons %
Peripherin	*63±1 *(59±1)	100 (100)	0 (0)	90±2 (85±3)	85±2 (76±4)
IB4	35±2 (36±2)	54±4 (57±1)	9±2 (11±2)	100 (100)	Not determined
CGRP	43±7 (28±3)	**55±9 **(34±3)	18±2 (19±4)	Not determined	100 (100)

Table 1. Quantitative analysis of neuronal markers and effect of ovariectomy. This table shows the percentages of total neurons, peripherin-positive neurons, peripherin-negative neurons, CGRP-positive neurons, or IB4-positive neurons that express peripherin, CGRP, or IB4. % Estrus (% OVX). There was a significant drop in the percentage of total neurons expressing peripherin and peripherin-positive neurons co-expressing CGRP following ovariectomy, $p=0.042$ and $p=0.049$ by student's t-test.

FIGURES

Figure 1.

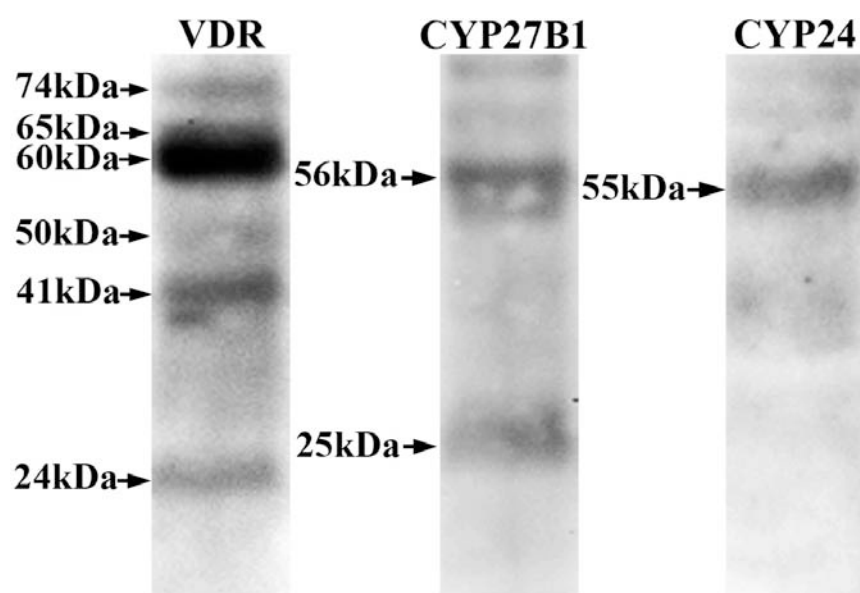


Figure 1. VDR, CYP27B1, and CYP24 proteins are present in adult DRG. Western blots of extracted DRG proteins from intact rats in estrus were probed for VDR, CYP27B1, or CYP24. Samples were run alongside molecular markers in order to estimate protein size. Images are cropped to show representative lanes.

Figure 2.

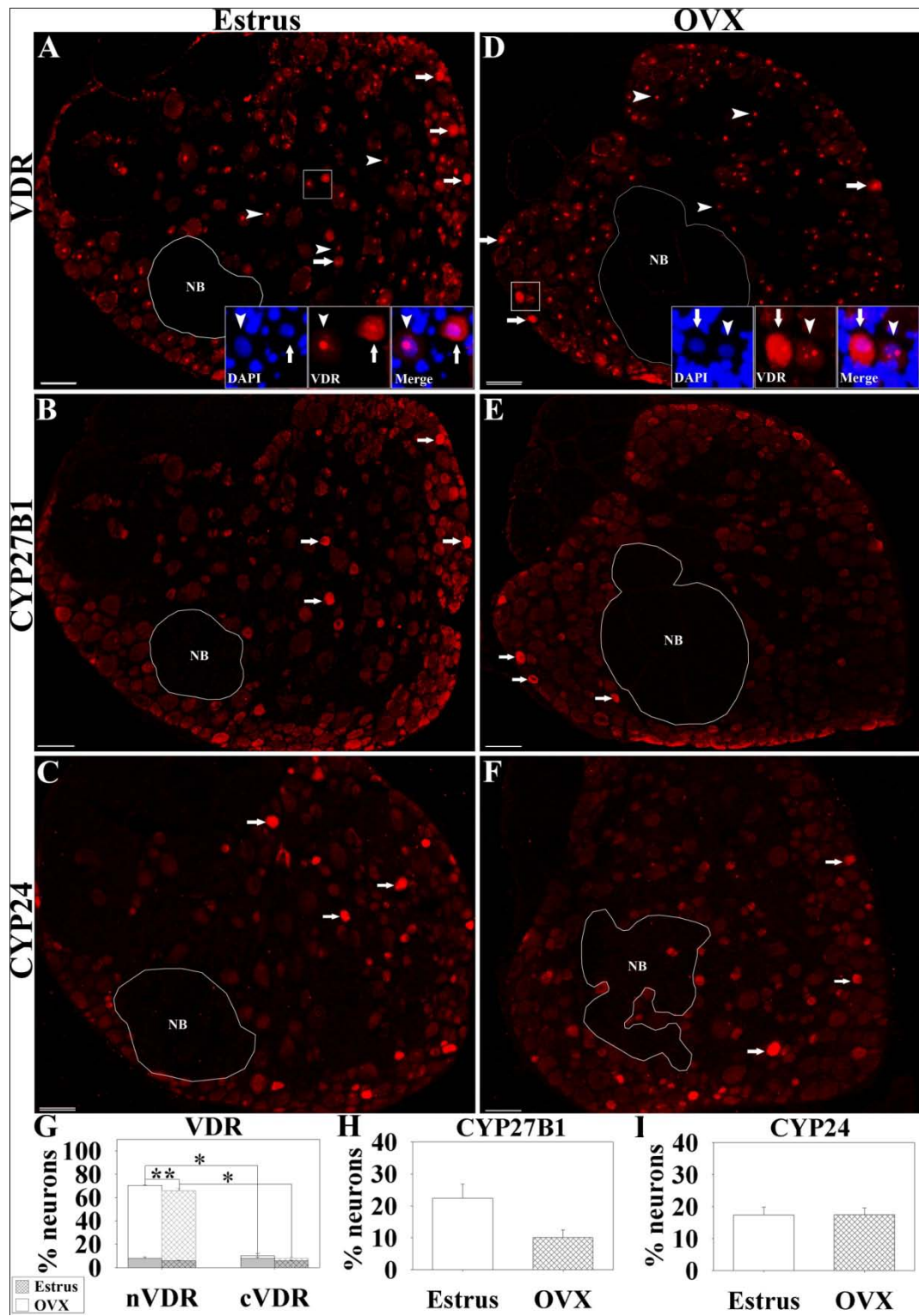


Figure 2. Cellular localization of VDR, CYP27B1, and CYP24 in adult DRG. Cryosections of L5 DRG from intact rats in estrus (A-C) or ovariectomized (OVX) rats (D-F) were immunofluorescently labeled for VDR (A&D; red), CYP27B1 (B&E; red), or CYP24 (C&F; red), and nuclei were stained with DAPI (shown only in the insets of A and D; blue). Examples of neurons with nuclear-localized VDR are indicated in A and D by arrow heads, and neurons with intense cytoplasmic VDR staining are indicated by arrows. The insets show magnified examples of neurons with nuclear or nuclear and cytoplasmic VDR immunoreactivity. In B& E, examples of neurons with intense labeling for CYP27B1 are indicated by arrows. In C & F, neurons with intense labeling for CYP24 are indicated by arrows. In A-F staining is not consistent with glial expression of VDR, CYP27B1, or CYP24 (ie. No staining surrounding neurons in satellite glia or in the Schwann cells of outlined nerve bundles (NB)). In G, the total proportion of neurons expressing VDR in estrus or after OVX is presented. The shaded area within each bar represents the proportion of neurons with both cytoplasmic and nuclear VDR staining. Significant differences between individual groups are shown by bracketing, $p = * < 0.001$ and $** 0.038$. The overall difference between Estrus and OVX expressing VDR was also significant, $p = 0.028$. H and I present quantitative analysis of the percentages of neurons immunostained for CYP27B1 and CYP24, respectively. Scale bar = 100 μ m (10 μ m in insets).

Figure 3.

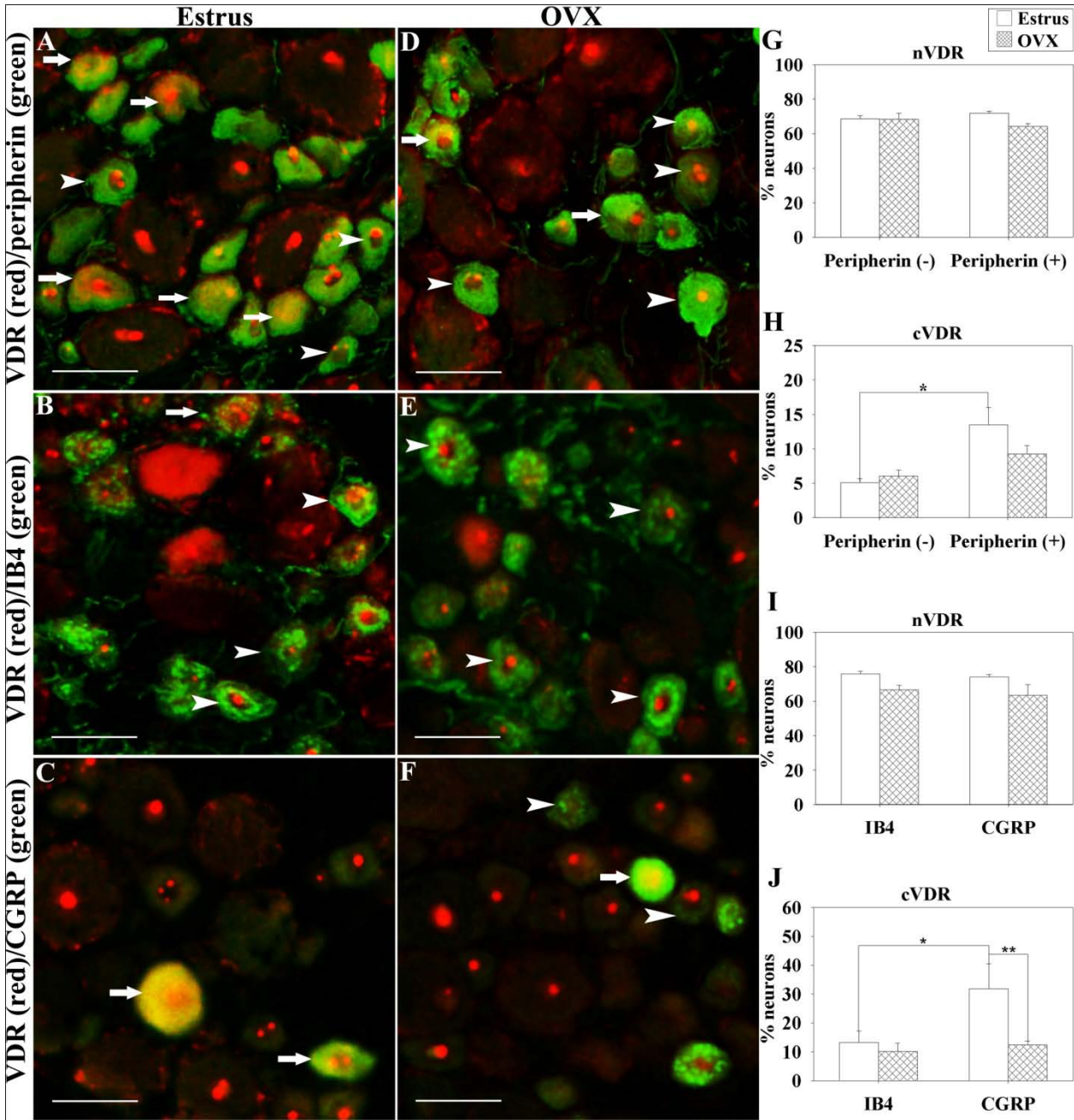


Figure 3. Cellular colocalization of VDR with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A-C) or ovariectomized rats (D-F) were immunofluorescently-labeled for VDR (A-F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A-F not shown). Neurons with nuclear VDR and neural markers are indicated by arrowheads, and neurons with cytoplasmic VDR and neural markers are indicated by arrows. G-J: The mean percentages of neurons with neural marker immunoreactivity that express nVDR or cVDR are presented in graphical form. Significant differences between individual groups are shown by bracketing, p= H) *0.002, J) *0.016 and **0.009. Overall differences between neural markers were significant for H) cVDR peripherin (+) vs. peripherin (-) (p=0.001) and J) cVDR IB4 vs. CGRP (p=0.039). Overall differences between Estrus and OVX were also significant J) p=0.027. Scale bar = 50µm.

Figure 4.

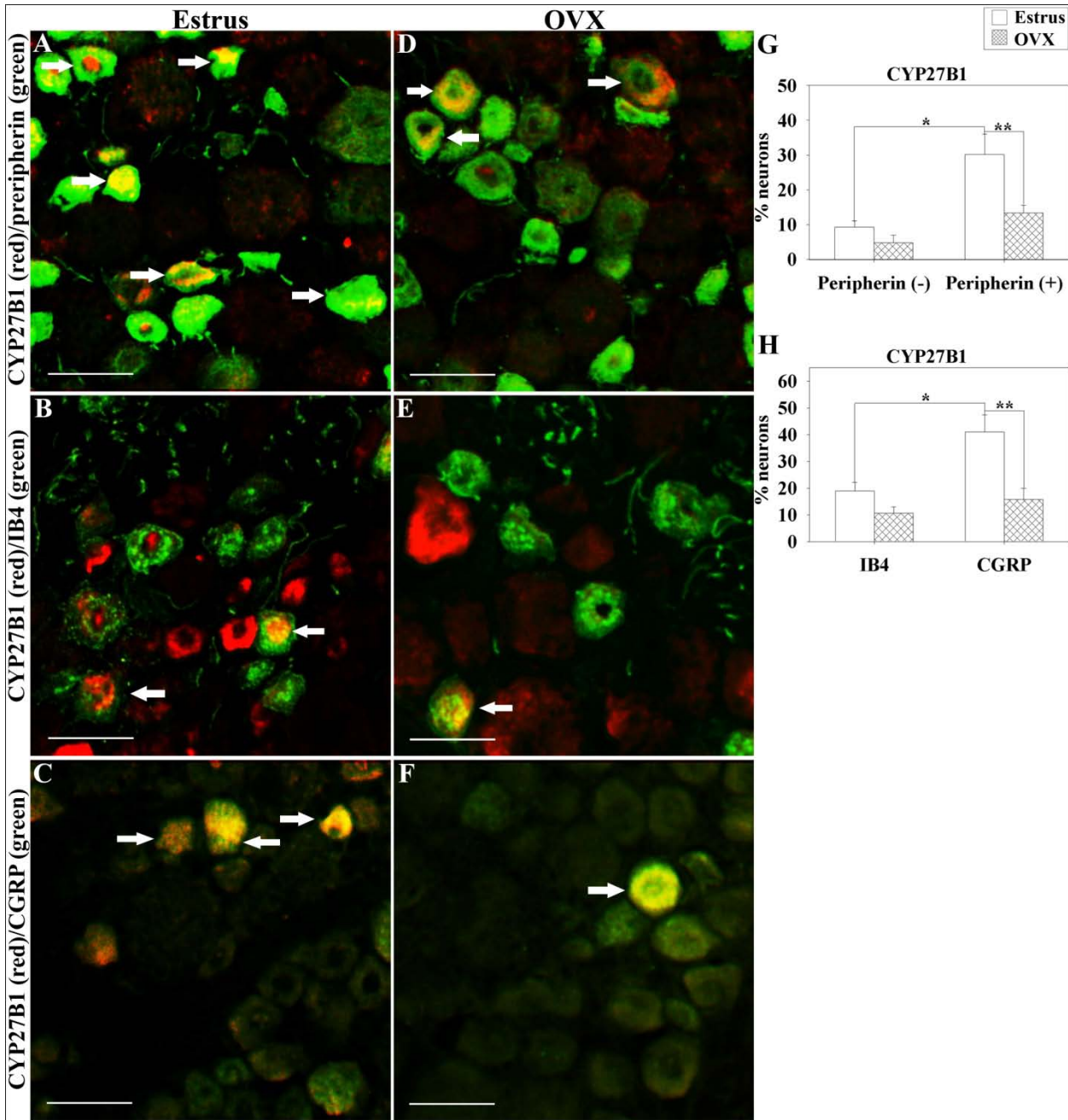


Figure 4. Cellular colocalization of CYP27B1 with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A-C) or ovariectomized rats (D-F) were immunostained for CYP27B1 (A-F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A-F not shown). Neurons with intense CYP27B1 and neural marker staining are indicated by arrows. G-J: The mean percentages of neurons with neural marker immunoreactivity that express CYP27B1 are illustrated in graphical form. Significant differences between individual groups are shown by bracketing, p= G) $* < 0.001$ and $** 0.003$, H) $* 0.003$ and < 0.001 . Overall differences between neural markers was significant for G) CYP27B1 peripherin (+) vs. peripherin (-) ($p < 0.001$) and J) CYP27B1 IB4 vs. CGRP ($p = 0.005$). Overall differences between Estrus and OVX were also significant G) $p = 0.005$ and J) $p = 0.001$. Scale bar = 50 μm .

Figure 5.

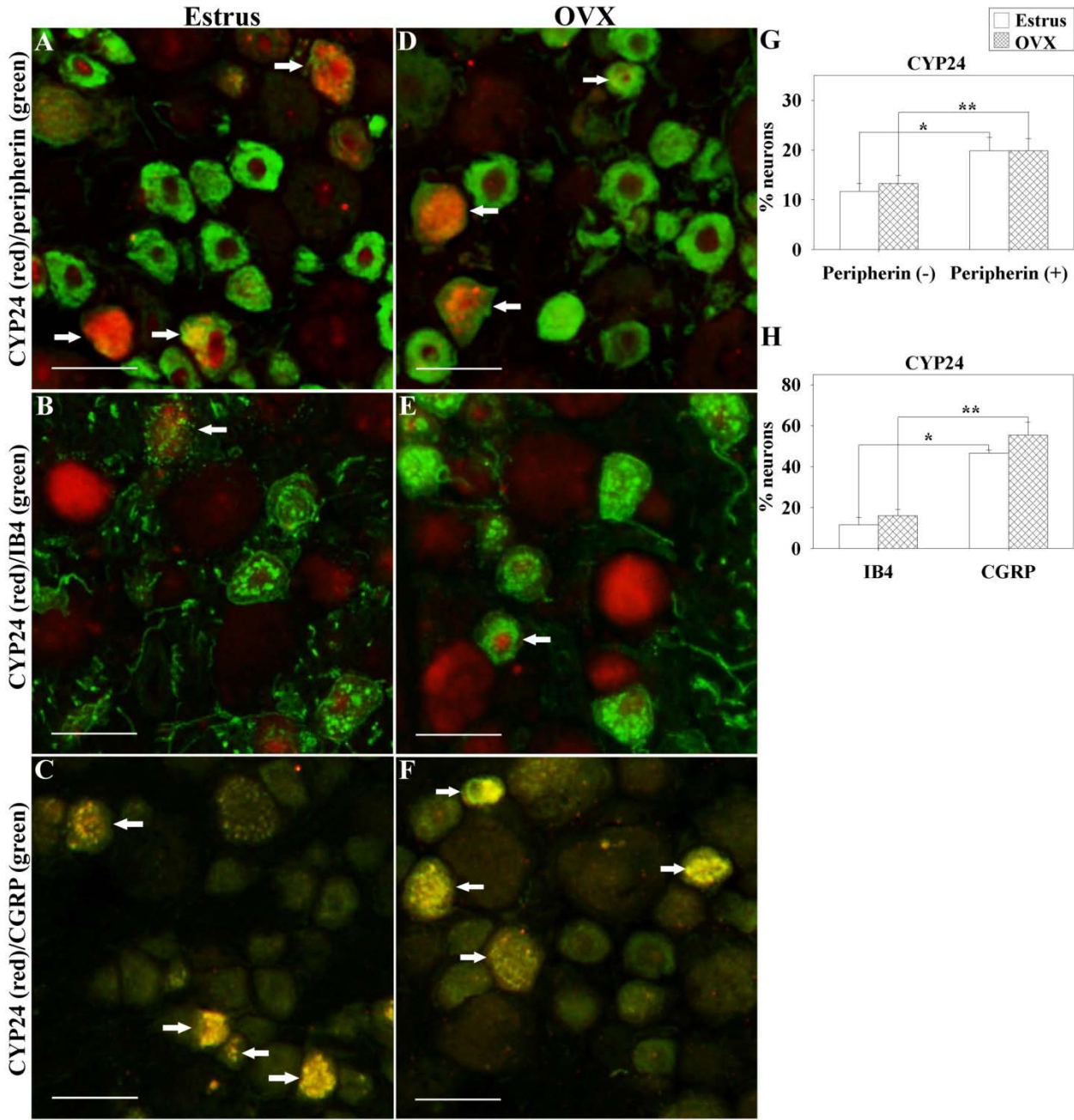


Figure 5. Cellular colocalization of CYP24 with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A-C) or ovariectomized rats (D-F) were immunostained for CYP24 (A-F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A-F not shown). Neurons with intense CYP24 and neural marker staining are indicated by arrows. G&H: The mean percentages of neurons with neural marker immunoreactivity that express CYP24 are illustrated in graphical form. Significant differences between individual groups are shown by bracketing, p= G) *0.024 and **0.005, J) * <0.001 and **0.001. Overall differences among neural markers were significant for G) CYP24 peripherin (+) vs. peripherin (-) (p=0.005) and J) CYP24 IB4 vs. CGRP (p <0.001). Scale bar = 50 μ m

Figure 6.

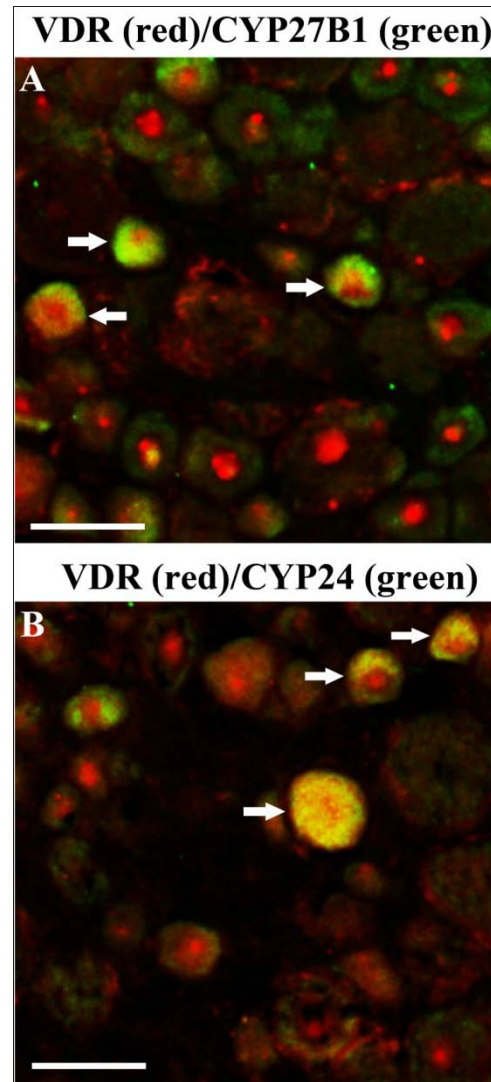


Figure 6. Cellular colocalization of VDR with the enzymes CYP27B1 or CYP24. Cryosections of L5 DRG from rats in estrus were immunostained for VDR (red) and the enzymes CYP27B1 (A) or CYP24 (B) (green) and nuclei were stained with DAPI (not shown). Neurons co-labeled for each enzyme and cVDR are indicated by arrows. Scale bar = 50 μ m.

Figure 7.

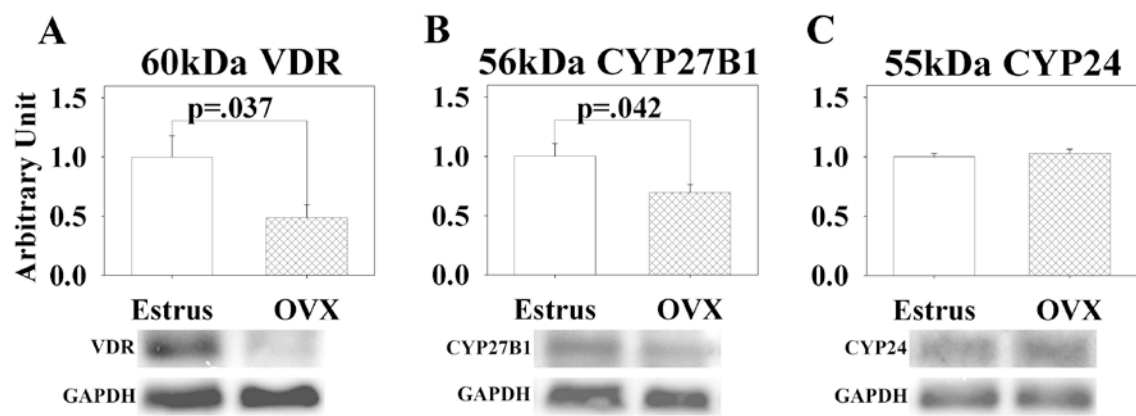


Figure 7. DRG expression of VDR and CYP27B1, but not CYP24 is reduced following ovariectomy. Western blots of proteins extracted from DRG of intact rats in estrus or ovariectomized rats were probed for VDR (A), CYP27B1 (B), or CYP24 (C) and GAPDH (A-C) as a loading control. A) The 60kDa band of VDR showed a significant decrease in intensity following ovariectomy. B) The 56kDa band of CYP27B1 also showed a significant drop in intensity following ovariectomy. C) There was no significant change in CYP24 after ovariectomy. OVX n=5, Estrus n=4, 2-3 replicates per animal were averaged. Significance determined by student's t-test.

CHAPTER 3. VITAMIN D DEFICIENCY PROMOTES SENSORY AXON SPROUTING AND MUSCLE HYPERSENSITIVITY

ABSTRACT

Musculoskeletal pain affects 35-50% of adults, most of whom are vitamin D deficient. Previous findings from this laboratory have demonstrated that putative nociceptors express vitamin D receptors (VDRs), suggesting that vitamin D metabolites can act directly on sensory nerves. In this study, 2-4 weeks of a vitamin D deficient diet produces deep muscle mechanical hypersensitivity in rats that is not corrected by dietary calcium supplementation. This hypersensitivity to deep pressure is accompanied by nociceptor hyperinnervation of the muscle. In culture, VDRs are enriched in small fiber sensory growth cones and rapid response receptors regulate neurite growth consistent with *in vivo* findings. Because vitamin D metabolites directly inhibit axonal growth of sensory c-fibers, vitamin D deficiency leads to muscle hyperinnervation, which may contribute to musculoskeletal pain.

INTRODUCTION

Vitamin D recently has been proposed to play wide ranging roles in human health, and deficiencies in this secosteroid are implicated in chronic musculoskeletal pain (Benson, Wilson et al. 2006; de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991; Heidari, Shirvani et al. 2010; Macfarlane, Palmer et al. 2005; McBeth, Pye et al. 2010; Plotnikoff and Quigley 2003) and an increased risk of falling (Annweiler, Montero-Odasso et al. 2010). 27.5-36% of adolescents and 35-50% adults suffer from chronic musculoskeletal pain (Bergman 2007; De Inocencio 2004) and up to 93% of them may be vitamin D deficient, with levels of serum 25-hydroxyvitamin D (25(OH)D) less than 20ng/ml (50nmol/L) (Plotnikoff and Quigley 2003). Some studies show that this pain may be relieved by vitamin D supplementation (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991). However, it is difficult to establish a causal relationship, as the incidence of musculoskeletal pain is increased in people that are older, have a higher body mass index, and spend less time out-of-doors, all factors contributing to vitamin D deficiency (Bergman 2007; Yetley 2008).

While vitamin D could affect pain sensitivity at multiple sites, we found recently that small unmyelinated dorsal root ganglion (DRG) sensory neurons express both nuclear and membrane/cytoplasmic vitamin D receptors (VDRs) (Tague and Smith 2010), suggesting that sensory neurons, including putative nociceptors, are direct targets of an active vitamin D hormone, 1,25-dihydroxyvitamin D (1,25(OH)₂D). In order to further investigate the relationship between vitamin D and musculoskeletal pain, a rat model in which vitamin D deficiency is reliably induced was used. Reducing dietary intake of vitamin D can induce persistent vitamin D deficiency in laboratory rats (Weishaar and Simpson 1987). However, vitamin D deficiency, in turn, leads to reductions in serum calcium that can affect many physiological functions independently (Johnson and DeLuca 2002; Uhland, Kwiecinski et al. 1992; Weishaar and Simpson 1987), although this can be corrected by administering a diet high in calcium and phosphate (Weishaar and Simpson 1987).

MATERIALS AND METHODS

Animals and diets

All animal protocols and procedures were in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the University of Kansas Medical Center Animal Care and Use Committee. Fifteen weaned (24 day-old) female Sprague Dawley (Harlan) rats were housed 2-3 per cage in forced-air-ventilated micro-isolation cages with a 14hr light/10hr dark light cycle and fed *ad libitum* a normal chow diet. At the age of 31 days, rats were anaesthetized with ketamine 70mg/kg (Pfizer)/xylazine 6mg/kg i.p. (Lloyd Laboratories) and ovariectomized via bilateral hindflank incision. Upon waking, and at 24hr and 48hr post-operation, rats were administered ketoprofen 5mg/kg s.c. (Ketofen; Fort Dodge Animal Health) as an analgesic. At 48 days of age, rats were randomly separated into three groups and fed one of three diets (blinded); **Control:** 2.2 IU/g vitamin D (cholecalciferol), 0.47% Ca, 0.3% P (Harlan Teklad, TD.07370), **VD-/+ Ca:** No vitamin D, 2.5% Ca, 1.5% P (Harlan Teklad, TD.07541), **VD-:** No vitamin D, 0.47% Ca, 0.3% P (Harlan Teklad, TD.89123). Food was provided in a ceramic bowl on the cage floor and replaced twice weekly. Rats were weighed weekly and food was weighed before and after changing (only data for week 0, 2, and 4 are presented in Table 1). Rats were maintained on their assigned diet for four weeks. After two weeks on the diet, one to two ml of blood was drawn from the tail vein. Serum was collected and stored at -20 °C. At the end of the study, rats were anaesthetized with ketamine 70mg/kg / xylazine 6mg/kg (i.p.), thoracic cavities were opened, blood was removed via cardiac puncture, and the rats were perfused with 50ml of cold 0.9% saline containing 10units/ml heparin (APP Pharmaceuticals) at a rate of 40ml/min, followed by 150-200ml of 4% formaldehyde (prepared in PBS from paraformaldehyde (Sigma-Aldrich)). Serum was collected and stored at -20C. See S1 for an overview of the experimental timeline.

Behavioral testing

All behavioral testing was performed in a quiet room under normal fluorescent lighting six to eight hours into the light cycle. The rat cages were moved to the behavior room 1hr before testing. All statistical

analysis of behavior was conducted using two way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons.

Muscle mechanical sensitivity- The muscle compression test used in this study is similar to that of models previously described (Sharma, Ryals et al. 2009; Skyba, Radhakrishnan et al. 2005). A digital Randall Selitto instrument (IITC Life Science Inc) with a plastic cap with a 0.5cm wide surface area (S2) was used. During the week prior to diet manipulation, rats were habituated to a rat sling (IITC Life Science, Inc) for 5min on two separate days. The day prior to starting the diets, and each week thereafter, rats were stabilized in the rat sling to allow free access to the hind limbs. While holding the left hind paw, the flat side of the probe was placed against the inner left calf, while the capped pressure sensor was placed on the center of the left exterior calf muscle. The instrument was slowly compressed until the rat attempted a limb withdrawal (or vocalized). The calf was compressed three times and the maximum force applied each time was recorded and averaged. To minimize stress, rats were restrained in the sling for no longer than 3min/week. To ensure that the same part of the calf muscle was compressed each time, the left calf was shaved each week 3 days before muscle compression under brief isoflurane anesthesia. During week four, after initial testing, 2.5% lidocaine/2.5% prilocaine cream (TOLMAR Inc.) was applied to the calf skin for 30 minutes and muscle sensitivity measurements were repeated. Similar to previous reports (Skyba, Radhakrishnan et al. 2005), there were no significant differences in muscle sensitivity before and after cutaneous lidocaine treatment (data not shown), verifying that this test measures deep tissue pressure sensitivity and not cutaneous mechanical sensitivity.

Cutaneous mechanical sensitivity- Cutaneous mechanical sensation was measured using a calibrated 4g monofilament. Rats were placed on a wire-mesh grid with plexiglass dividers and allowed to habituate for 20min. The monofilament was applied to the plantar surface of the hind paw five times per trial for a total of three trials. The number of positive responses was recorded, and the percent withdrawal response for each paw was calculated. A response was considered positive when the rat retracted its hind paw in

response to the monofilament. The results shown are averages from the left and right hind paws.

Cutaneous sensitivity was measured two days before starting diet manipulation and each week thereafter.

Balance and motor co-ordination - An elevated, 2.5 cm wide round dowel was suspended between two support stands with an enclosed plastic platform at the one end. Rats were trained at the ages of 28, 39, and 41 days to walk across the beam into the platform box, by placing them on the beam at increasing distances from the box, until they voluntarily walked the full 110cm of the beam. Two days before starting the diets, and at week 2 and 4, rats were videotaped for three beam walk trials. The videos were analyzed to determine the number of hind paw footfall slips per beam walk, which were then averaged.

Muscle weakness - - Three days before starting the diets, and at weeks 2 and 4, rats were placed on a wire grid attached to a digital force gauge (San Diego Instruments). The maximum grip force applied by the forelimbs was measured as the rats were gently pulled by the base of their tail until their grip was broken. This was repeated three times for each rat and the maximum force was recorded.

General locomotor activity - Rats were placed in a Force Plate Actimeter (BASi) (Fowler, Birkestrand et al. 2001) and allowed to move freely for 5min. Movements were detected by force transducers and recorded by a computer and rearing events were recorded by the investigator.

Serum Measurements

Serum 25-hydroxyvitamin D was measured by direct ELISA (Immunodiagnostik) according to manufacturer instructions. Serum calcium and phosphorous was measured by an outside laboratory (Physicians Reference Laboratory, L.L.C) by spectrophotometry.

Tissue processing

The left calf muscles (gastrocnemius and soleus) were removed and post-fixed in Zamboni's fixative overnight at 4°C. Weight and volume (determined by fluid displacement in a graduated cylinder) were

recorded. The muscles were washed for two weeks and cryoprotected at 4°C in phosphate buffered saline containing 30% sucrose changed daily. The entire calf was cut in the transverse plane at 25%, 50%, and 75% of the way through the muscle. The muscle was embedded in tissue freezing media (Electron Microscopy Science), frozen in liquid nitrogen-cooled isopentane, and stored at -80°C.

The left tibia was removed and post-fixed in Zamboni's fixative overnight at 4°C. The bones were washed at 4°C in PBS changed daily for three days, then decalcified in PBS containing 10% EDTA for two weeks at 4°C. The decalcified bones were then cryoprotected overnight at 4°C in PBS containing 30% sucrose. The bone was cut in half along the transverse plane. The proximal portion was cut in half along the sagittal plane and the medial portion was embedded in tissue freezing media for sagittal sectioning of the epiphyseal plate. The distal half of the bone was embedded in tissue freezing media for transverse sections of the diaphysis. Bones were frozen on dry ice and stored at -80°C.

Immunostaining of nerve fibers in muscle

Thawed 20µm cryosections of muscle were immunofluorescently labeled. Slides were incubated in pre-incubation solution (1.5% donkey serum (Jackson ImmunoResearch), 0.5% gelatin (Sigma-Aldrich), and 0.5% Triton X-100 (Sigma-Aldrich) prepared in Superblock (Thermo Scientific) for 1hr at room temperature. Primary and secondary antibodies were diluted in incubation solution (50% preincubation solution, 50% Superblock). Primary antibodies were incubated overnight at room temperature and secondary antibodies incubated for two hours. Before and after the secondary antibody, slides were washed in PBS containing 0.25% Triton-X 100. Slides were then dipped in distilled water and mounted with coverslips using 50% glycerol in PBS. Primary and secondary antibodies were diluted as follows: 1:500 chicken anti-peripherin (Chemicon), 1:200 sheep anti-CGRP (Biomol), 1:1,000 rabbit anti-VMAT2 (Chemicon), or 1:1,500 rabbit anti-NFH (Sigma), 1:1,500 donkey anti-chicken DyLight 488 (Jackson), 1:750 Donkey anti-sheep Dylight 649 (Jackson), 1:1,000 donkey anti-rabbit 647 (Invitrogen).

Quantitation of innervation density

Images were taken (blinded) from transverse sections of the left lateral gastrocnemius muscle, which is located on the outside of the calf where most of the force was applied during muscle compressions. A total of 18 images were taken per animal, 6 each from three levels at 25%, 50%, and 75% through the proximo-distal extent of the muscle using a Nikon 80i epifluorescent microscope. A stereological grid was superimposed over the images, and numbers of grid intersections overlying immunofluorescently-labeled axons was counted and divided by the total number of tissue intersections, to provide the apparent percentage area occupied by nerves (Clarke, Bhattacharjee et al. 2010). Statistical analysis was conducted using one way ANOVA and Student-Newman-Keuls multiple comparisons.

Bone measurements

Hematoxylin and eosin-stained 20 μ m sections of bone were analyzed. For growth plate analysis, three fully visible chondrocyte columns in the center of each section were analyzed for height and cell number and averaged per section. The transverse bone sections of the diaphysis were analyzed for bone circumference and bone marrow area. Statistical analysis was completed with one way ANOVA and Student-Newman-Keuls multiple comparisons.

Primary neuronal cell cultures

Sensory: DRG were removed from six week old female Sprague Dawley rats, dissociated with 2 units/ml dispase (ICN Biomedicals Inc) and 2mg/ml collagenase type 1A (Sigma) and plated on glass coverslips (Bellco) coated with poly-D-lysine (Sigma) and laminin (Invitrogen) in 24-well culture dishes. Cultures were maintained in Neurobasal A media (Invitrogen) containing 1mM glutamine (Sigma), 2% B27 (Gibco), 100 μ g/ml primocin (InvivoGen), 20 μ M FrdU (Sigma) and 20 μ M uridine (Sigma) for 4 days, with concentrations of 1,25(OH)₂D₃ or JN that ranged from 0-100pM. Cultures were incubated at 37°C with 5% CO₂ and media was changed after 24hrs and 72hrs. Cultures were fixed in 4% formaldehyde for

one hour, DAPI stained, and immunofluorescently-labeled for peripherin (chicken 1:1000, Chemicon), NFH (mouse 1:200, Sigma), or VDR (rabbit 1:25, GeneTex) peripherin and GAP43 (mouse 1:240, Zymed). Cultures were grown in triplicate and repeated twice.

Sympathetic: Superior cervical ganglia were removed from one day-old rats, dissociated with 0.25% trypsin-EDTA (Gibco) and collagenase 1A (Sigma) and plated on poly-D-lysine and laminin-coated coverslips. Cultures were maintained in Neurobasal A media as described above with the addition of 10µg/ml NGF (Alomone Labs) for 48 hours, with 0, 20pM or 100pM of 1,25(OH)₂D₃. Cultures were fixed in 4% formaldehyde, DAPI stained and immunofluorescently-labeled for peripherin and VDR. Cultures were grown in triplicate and repeated twice.

Neurite area quantitation

A stereological grid was superimposed over culture images and the number of times a grid intersection crossed an immunofluorescently-labeled nerve fiber was counted and divided by the total number of intersections within each field to provide neurite area; this was divided by the number of viable neurons, determined by DAPI staining, to provide an estimate of neurite area per neuron. Statistical analysis was completed with one way ANOVA and Student-Newman-Keuls multiple comparisons.

RESULTS AND DISCUSSION

To assess how vitamin D deficiency alters deep muscle sensitivity to noxious mechanical stimuli, Sprague Dawley rats were fed chow deficient in vitamin D, with or without high levels of calcium. Starting at seven weeks of age, rats were fed *ad libitum*, for four weeks, one of three blinded diets: control, vitamin D deficient with increased calcium and phosphate (VD-/Ca), or vitamin D deficient with normal levels of calcium and phosphate (VD-) (Table 1). The VD-/Ca diet was based on previously published reports that increasing dietary Ca from 0.47% to 2.5% normalizes serum Ca in vitamin D

deficiency (1.5% P is needed as a counterbalance)(Weishaar and Simpson 1987). Female rats were ovariectomized 2.5 weeks prior to initiation of the diet to remove the influence of cycling ovarian hormones on behavioral sensitivity testing (Craft 2007) and VDR expression in sensory nerves (Tague and Smith 2010). Additionally, the incidences of vitamin D deficiency and musculoskeletal pain are high in women and tend to be associated with suppressed ovarian hormones (i.e. post-menopause, pharmacologically-induced estrogen suppression) (Alexander, Dennerstein et al. 2007; Gaugris, Heaney et al. 2005; Khan, Reddy et al. 2010). Rats were housed under normal fluorescent lighting with a 14 hr light/10 hr dark cycle. Rats fed vitamin D-deficient diets showed weight gains comparable to control subjects over the 4-week study (Table 1). However, food consumption was increased in the VD-/Ca group throughout the study and in the VD- group at week four (Table 1). To confirm vitamin D deficiency, blood was drawn at 2 weeks from the tail vein and at 4 weeks via cardiac puncture. 25(OH)D serum concentrations in both VD-/Ca and VD- rats were reduced below 25nmol/L by week 2 and below 10nmol/L by week 4 (Table 1). In addition, serum Ca and P levels at week four were not reduced even though Ca is reported to be decreased in VD- rats after longer intervals (Weishaar and Simpson 1987).

To determine whether vitamin D deficiency causes increased mechanical muscular sensitivity, we used a behavioral testing paradigm developed for this purpose (Sharma, Ryals et al. 2009; Skyba, Radhakrishnan et al. 2005). Rats were placed weekly in a restraining sling allowing free access to their limbs. While holding the left foot, the left calf muscle was compressed using a modified digital Randall-Selitto testing device (S2) until the rat attempted to withdraw the limb or vocalized withdrew (or vocalized), and the maximum force applied by the testing device was recorded. Between 0 and 2 weeks, the force required to elicit a response increased in control rats, a finding consistent with increased body mass occurring in this period (Table 1), and plateaued thereafter (Fig. 1A). In VD- rats, mechanical sensitivity was normal through week 2 of the diet, but was increased by week 3 and was significantly higher than controls by week 4 (Fig 1A). Rats receiving the vitamin D-deficient diet with elevated Ca showed normal sensitivity at week 1, but marked hypersensitivity by week 2 and thereafter (Fig. 1A). Thus, the high calcium diet not only failed to reverse the hyperalgesic phenotype; it accelerated the onset of deep tissue hypersensitivity. These findings support the hypothesis that dietary vitamin D deficiency produces muscle hypersensitivity within 2-3 weeks in rats.

Gloth et. al first described patients with vitamin D deficiency attributed pain, which was triggered by minimal movement or touch (Gloth, Lindsay et al. 1991). Accumulating evidence supports a causal role for vitamin D deficiency in some (Benson, Wilson et al. 2006; de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991; Heidari, Shirvani et al. 2010; Macfarlane, Palmer et al. 2005; McBeth, Pye et al. 2010; Plotnikoff and Quigley 2003), but not all (e.g. fibromyalgia (Block 2004; de Rezende Pena, Grillo et al. 2010; Heidari, Shirvani et al. 2010; Tandeter, Grynbaum et al. 2009; Warner and Arnsperger 2008)) types of musculoskeletal pain. However, it is unclear whether hypersensitivity is generalized. To determine if hypersensitivity was apparent in other modalities, we evaluated cutaneous sensitivity of the hind paw plantar surface using a von Frey calibrated 4g monofilament. The percentage of applied mechanical stimuli that elicited a withdrawal response was assessed on a weekly basis. There were no differences between groups in cutaneous sensitivity at any week (Fig 1B). Therefore, while a vitamin D deficiency resulted in deep tissue mechanical hypersensitivity, this does not appear to be due to wide-spread changes in sensory threshold that affect cutaneous mechanical withdrawal latencies.

Changes in muscle sensitivity could affect other types of behavior, including balance and coordination. Accordingly, vitamin D deficient rats were tested for normal balance in a beam-walk test. Rats were trained to walk along a 110cm long, 2.5cm diameter elevated beam to an enclosed platform. At week 0, week 2, and week 4, this activity was video-recorded and analyzed to determine the number of times that their footfalls slipped off the beam. Rats receiving a normal diet showed substantial improvement in traversing the beam between 0 and 2 weeks, and slips remained low at 4 weeks (Fig. 1C). Rats receiving the VD- diet, however, failed to show improvement at 2 weeks or 4 weeks. Rats receiving the vitamin D deficient diet supplemented with Ca performed worse than either control or VD- groups at week 2 and failed to improve during the course of the study. Because the vitamin D deficient rats, especially the VD-/Ca rats, tended to sit lower on the beam, use more caution, and make more balance corrections, these data may underestimate the true extent of motor coordination deficits evoked by vitamin D deficiency.

The balance deficits observed in vitamin D deficient rats could be secondary to alterations in sensory innervation, but may also be due to myopathic changes in muscle that occur with protracted

vitamin D deficiency (Schott and Wills 1976). To assess any overt muscle dysfunction, we conducted grip strength analyses (Fig. 1D). Forelimb grip strength increased with age in all groups, and vitamin D-deficient rats showed no deficits relative to rats on normal diets, indicating an absence of gross muscle dysfunction. To determine whether the apparent balance deficits were due to problems in mobility, the general locomotor activity of each rat was analyzed in a force-plate actimeter (BASi). There were no significant differences in the overall locomotor activity between treatment groups, including total distance traveled (Fig. 1E) and number of rearing events (Fig. 1F). Thus the balance deficits do not appear to result from grossly limited locomotor mobility or overt muscle weakness.

Because vitamin D deficiency and deep muscle hypersensitivity are well established by four weeks after the initiation of vitamin D deficient diets, we examined tissues at this time. Calf muscle volume (Fig. 2A) and weight (not shown), were essentially identical in all groups, corroborating measurements of muscle strength and confirming an absence of measurable muscle atrophy in the vitamin D-deficient animals at this stage. It has been argued that musculoskeletal pain in vitamin D deficiency is caused by the expansion of uncalcified bone matrix, resulting in pressure on the richly innervated periosteum (Mascarenhas and Mobarhan 2004). Despite normal serum calcium, prolonged vitamin D deficiency in rats can result in shortened and bent tibia with enlarged and disorganized epiphyseal growth plates (Lester, VanderWiel et al. 1982). However, in the current study, tibiae from the vitamin D-deficient rats showed no overt abnormalities or differences in size or shape relative to controls. Histological analyses of decalcified tibial sections also showed organized growth plates with no expansion, either by height or number of chondrocytes per cartilage column (S3 and S4). No changes in diaphysis circumference or marrow area were present (S4). These findings comport with observations that muscle symptoms in vitamin D deficiency occur prior to bone pathology (Masood, Narang et al. 1989), and suggest that gross changes in muscle or bone integrity do not underlie deep muscle pain in the early stages of vitamin D deficiency.

There is growing evidence that many clinical pain syndromes are accompanied by nociceptor axon sprouting within affected peripheral tissues (Alfredson, Ohberg et al. 2003; Bohm-Starke, Hilliges et al. 1999; Schubert, Weidler et al. 2005). Because steroid hormones other than vitamin D can influence sensory axonal outgrowth (Blacklock, Johnson et al. 2005), we examined innervation of control and

vitamin D deficient muscle. Sections were immunostained for peripherin, a marker used to identify intact unmyelinated axons (Goldstein, House et al. 1991). VD-/Ca and VD- rats showed nearly a twofold increase in peripherin-immunoreactive (-ir) nerve density relative to controls (Fig. 2B, 3A-C). To identify which populations of c-fibers are increased in vitamin D-deficiency, sections of muscle were immunostained for calcitonin gene related peptide (CGRP) as a marker for 'peptidergic nociceptors' (Lawson 1992), and vesicular monoamine transporter 2 (VMAT2) as a marker for noradrenergic sympathetic axons (Headley, Suhan et al. 2007). CGRP-ir axon density was increased approximately twofold (Fig. 2C, 3D-F) while that of VMAT2-ir was not significantly changed (Fig 2D and 3G-I). To address whether the myelinated fibers innervating the muscle are affected by vitamin D deficiency, muscle sections were immunostained for neurofilament H (NFH), a marker of large myelinated motor and proprioceptive fibers, and bungarotoxin, which reveals terminal muscle innervation by staining motor end plates. We found no significant changes in sectional area of NFH-ir (Fig 2E and 3J-L) or bungarotoxin between control and vitamin D deficient muscles. These findings indicate that vitamin D deficiency leads to selective muscle hyperinnervation by unmyelinated fibers, which are most likely peptidergic nociceptors.

We recently reported that adult sensory dorsal root ganglion neurons contain vitamin D receptors and metabolic enzymes (Tague and Smith 2010). Accordingly, vitamin D deficiency could affect muscle innervation through a direct action on DRG neurons. To test this hypothesis, adult DRG neurons were grown *in vitro*. The expression of VDRs by cultured neurons was first verified; results reveal VDR localized to the nucleus and somal cytoplasm (Fig. 4A, B). Interestingly, VDR was concentrated within growth cones of peripherin-ir neurites, and co-localized with the growth associated protein GAP43 (Fig. 4C-E), suggesting a role for VDR in growth cone kinetics.

To determine if vitamin D levels influence unmyelinated sensory axonal architecture, DRG neurons were cultured with various concentrations of 1,25(OH)₂D₃. The normal mean serum concentration of 1,25(OH)₂D is approximately 80pM (Lund and Sorensen 1979), and patients with severe musculoskeletal pain are reported to have levels ranging from 12pM to 45pM (Gloth, Lindsay et al. 1991). To mimic this range, 1,25(OH)₂D₃ concentrations between 0 and 100pM were added to primary DRG cultures. DRG neurons grown in concentrations of 1,25(OH)₂D₃ between 60pM and 100pM,

(corresponding to normal serum levels) showed typical axon outgrowth (Fig. 4F&I). Below 60pM there was a steady increase in neurite outgrowth until 20pM, where outgrowth peaked at 60% greater than controls (Fig. 4G, I). These findings indicate that 1,25(OH)₂D₃ can have direct effects on the outgrowth of sensory unmyelinated neurons. At normal physiological levels it inhibits neurite outgrowth, and therefore correlates with the *in vivo* increases in muscle innervation during vitamin D deficiency.

We assessed whether vitamin D deficiency promotes growth of other peripheral nerve axons by examining whether superior cervical ganglion (SCG) neurons are affected by 1,25(OH)₂D₃. The expression of VDR in sympathetic neurons *in vivo* and in culture was first assessed. Similar to small-fiber DRG neurons, VDR-ir was present in both cytoplasmic and nuclear compartments of SCG neurons (S5 A&C). However, while VDR was concentrated at the growth cone of sensory neurites, it appeared evenly distributed throughout sympathetic neurites, and was not enriched in the axon terminal (S5 C&E). SCG neurons were cultured with 0pM, 20pM, or 100pM 1,25(OH)₂D₃. In contrast to small-diameter sensory neurons, 1,25(OH)₂D₃ had no effect on peripherin-ir neurite outgrowth from sympathetic neurons (S5 F). This is consistent with *in vivo* findings demonstrating that VMAT2-ir axon density did not change in muscles of rats with vitamin D deficiency, and supports the hypothesis that vitamin D deficiency produces selective effects on c-fiber sensory neurons.

The vitamin D-induced outgrowth of sensory but not sympathetic axons suggests that vitamin D signaling may differ between these two types of neurons. VDRs can signal through either classical genomic or rapid response pathways via membrane-bound/cytoplasmic receptors that modulate ion flux and generation of second messengers (Mizwicki and Norman 2009). The enrichment of VDR in growth cones of sensory but not sympathetic axons is consistent with the latter rapid signaling mechanism in regulating axonal outgrowth. To determine if sensory axon outgrowth is regulated by rapid membrane/cytoplasmic signaling mechanisms, we used 6-s-cis lumisterol (JN), which selectively binds to and activates membrane VDRs but has no effect on nuclear VDR signaling (Norman, Okamura et al. 1997). JN was applied to DRG cultures in the same concentrations as 1,25(OH)₂D₃ in the previous experiment. Immunostaining for large diameter NFH-ir neurons, which *in vivo* primarily express only nuclear VDRs (Tague and Smith 2010), showed no effect of JN on outgrowth, indicating that large diameter neurons fail to respond to membrane/cytoplasmic VDR activation with increased outgrowth

(Fig. 4J). However, JN did elicit neurite outgrowth of peripherin-ir fibers at concentrations similar to those of $1,25(\text{OH})_2\text{D}_3$, with peak outgrowth also occurring around 20pM (Fig. 4J). Therefore, VDR rapid response mechanisms likely regulate neurite outgrowth from sensory c-fiber neurons.

Musculoskeletal pain affects up to 50% of adolescents and adults (Bergman 2007; De Inocencio 2004) and is the second leading cause of lost productivity due to pain (Stewart, Ricci et al. 2003), making this an important clinical problem. Vitamin D supplementation is reported to be a safe and effective approach for reversing this pain in some individuals (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991; Heaney 2008). Our findings show that rats fed a vitamin D-deficient diet also have deep muscle pain, providing evidence for a role of vitamin D in some muscle pain syndromes.

Hypovitaminosis D-induced mechanical hypersensitivity was not secondary to, nor exacerbated by hypocalcemia in our model. In fact, high dietary calcium concentrations appeared to accelerate vitamin D deficiency-induced increases in deep tissue sensitivity. In vitamin D deficiency, hypocalcemia can lead to increased parathyroid hormone release, which increases CYP27B1 activity resulting in greater conversion of $25(\text{OH})\text{D}$ to $1,25(\text{OH})_2\text{D}$. In fact, when serum $25(\text{OH})\text{D}$ levels drop below 40nM, $1,25(\text{OH})_2\text{D}$ levels often increase (Need, Horowitz et al. 2000). However, this is not always the case, as all patients with pain in the study by Gloth et. al. had $1,25$ -dihydroxyvitamin D_3 serum concentrations below 45pM (Gloth, Lindsay et al. 1991). All of the patients tested in that study had normal serum calcium levels, and most were reportedly taking calcium supplements or multivitamins. In rats, elevated dietary calcium inhibits CYP27B1 expression and attenuates the initial spike in serum $1,25(\text{OH})_2\text{D}$, and accelerates its reduction (Anderson, Lee et al. 2010; Mallon, Boris et al. 1981), and may well account for the more rapid onset of muscle sensitivity in our rats receiving calcium-enriched chow. In view of how common dietary calcium supplementation is, this may be a clinically important factor modulating muscle sensitivity in vitamin D-deficient patients.

The finding that the affected tissue also shows selective hyperinnervation by putative nociceptors is in accord with reports in which hypersensitivity is accompanied by abnormal increases in numbers of presumptive pain-sensing nerves (Alfredson, Ohberg et al. 2003; Bohm-Starke, Hilliges et al. 1999; Schubert, Weidler et al. 2005). While the contribution of increased innervation to pain sensitivity remains

unclear, actively growing axons show greater excitability than do quiescent axons (Janig, Grossmann et al. 2009), as do neurons with more complex axonal geometries (Janse, Peretz et al. 1999). Similarly, more extensive axonal branching is likely to result in greater summation of locally evoked depolarizing potentials. Accordingly, the finding that low vitamin D levels induce sensory c-fiber sprouting is consistent with the increased muscular sensitivity observed in vitamin D deficient rats. Regarding locomotor activity and balance, the current observations are consistent with a clinical study, showing that while low vitamin D levels were associated with increased fall risk, they were not associated with decreased grip strength or gait speed (Faulkner, Cauley et al. 2006). Whether increased mechanical muscle sensitivity also contributes to the balance deficits and increased risk of falling in vitamin D-deficient humans (Annweiler, Montero-Odasso et al. 2010) remains to be determined.

Vitamin D metabolites are known to regulate nervous system development (Levenson and Figueiroa 2008), but a role in postnatal sensory axon growth has not been reported previously. In this regard, vitamin D deficiency appears to be selective to c-fiber sensory neurons, as reduced $1,25(\text{OH})_2\text{D}_3$ levels did not increase neurite growth by sympathetic neurons or large fiber sensory neurons. Hypovitaminosis D-induced sensory axon sprouting appears to involve signaling by membrane/cytoplasmic rapid response VDRs. Rapid VDR signaling can affect intracellular calcium mobilization (Norman, Okamura et al. 1997), and growth cone calcium homeostasis is known to influence growth cone kinetics (Gomez and Zheng 2006). Thus, VDR-mediated regulation of growth cone calcium channels represents a likely mechanism mediating sensory axon outgrowth. While we are just beginning to explore the details of this phenomenon, the implications are exciting, as unlike many other pain therapies, proper vitamin D supplementation is safe, inexpensive, and has virtually no side-effects (Heaney 2008).

TABLES

Table 1.

	Diet			Food Intake		Weight			Serum 25D		Serum Ca	Serum P
	Vitamin D3 (IU/g)	Ca (%)	P (%)	Week 2 (kcal/day)	Week 4 (kcal/day)	Week 0 (g)	Week 2 (g)	Week 4 (g)	Week 2 (nmol/L)	Week 4 (nmol/L)	Week 4 (mg/dL)	Week 4 (mg/dL)
Control	2.2	0.47	0.3	58.9±0.4	63.7±0.1	164±4	224±5	264±5	51.8±6.0	70.8±6.3	11.0±0.1	8.5±0.4
VD-/+ Ca	-	2.5	1.5	64.1±1.3*	67.8±1.9*	168±4	225±6	262±9	24.6±5.5*	6.6±0.7*	11.0±0.1	8.3±0.4
VD-	-	0.47	0.3	59.9±0.3	66.4±0.6*	166±3	224±2	260±5	18.3±2.5*	4.2±1.5*	11.0±0.1	8.8±0.4

*p<0.05 compared to control. Values shown are means±SE

Table 1. Diet, weight, and serum analysis.

Table 2.

	Growth Plate		Diaphysis	
	Thickness (μm)	Cell #/ column	Circumference (mm)	Marrow area (mm^2)
Control	282 \pm 20	20.8 \pm 0.9	6.9 \pm 0.1	3.5 \pm 0.1
VD-/ +Ca	274 \pm 25	20.0 \pm 1.0	7.1 \pm 0.4	3.6 \pm 0.3
VD-	279 \pm 14	21.1 \pm 1.0	6.8 \pm 0.4	3.5 \pm 0.2

Table 2. No changes in bone parameters were found in rats fed a vitamin D deficient diet for four weeks.

FIGURES



Figure 1. Experimental timeline. Experimental procedures are indicated on a timeline of rat age.

Figure 2.



Figure 2. Modification of the digital Randall Selitto instrument (IITC Life Science Inc). A plastic cap with a 0.5cm diameter surface area was placed over the pressure sensor.

Figure 3.

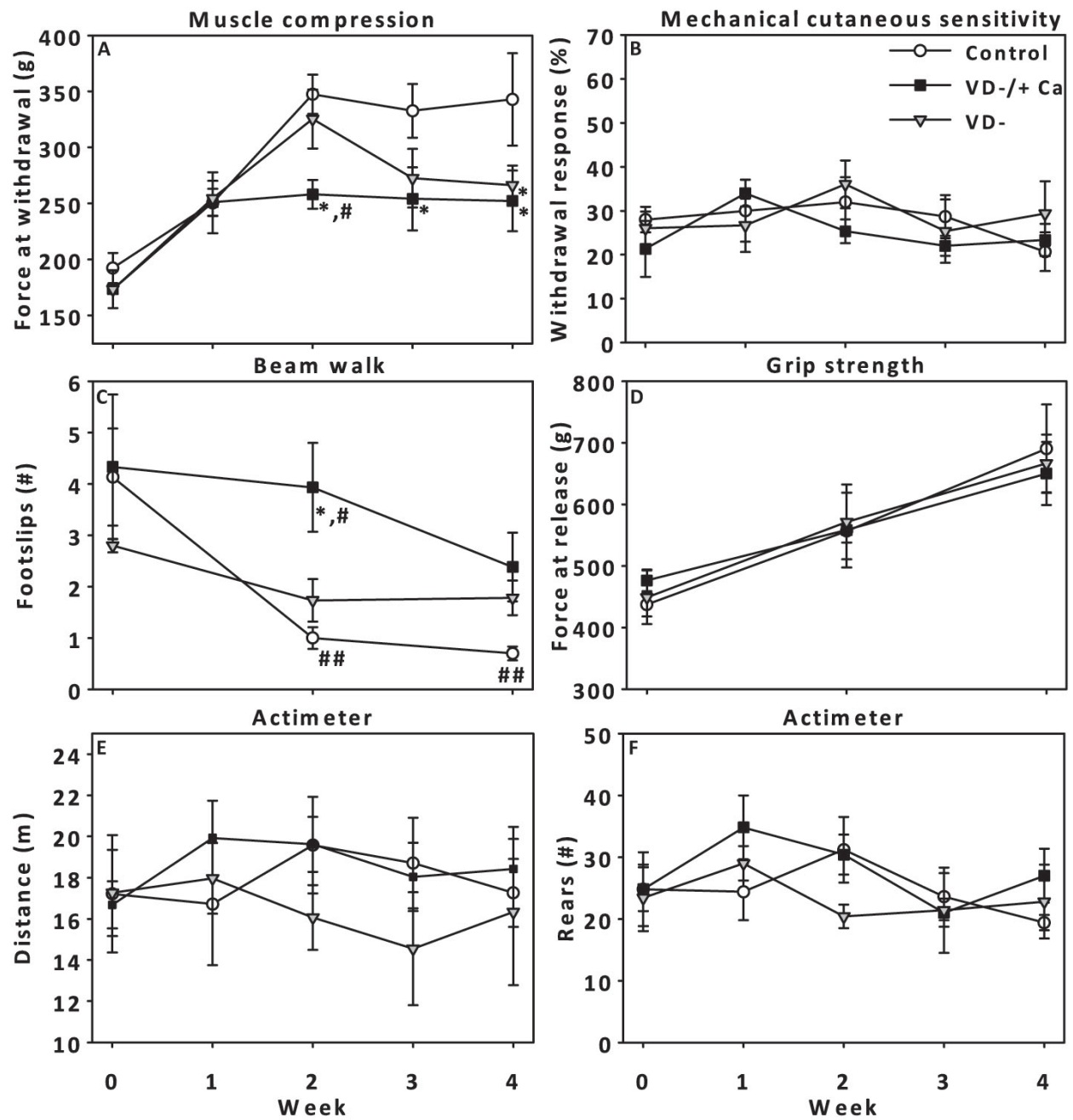


Figure 3 Behavioral comparisons of control (○), VD-/Ca (■), and VD- (▼) rats. **(A)** Changes in deep tissue pressure sensitivity, measured as the maximum force (g) required to elicit an attempted limb withdrawal or vocalization to mechanical compression of the calf muscle. **(B)** There were no changes in cutaneous mechanical sensitivity as measured by the percentage of withdrawal responses to the application of a 4g monofilament to the plantar surface of the hind paw. **(C)** Changes in balance were measured by the number of hind paw slips while traversing an elevated 2.5cm diameter 110cm long beam. VD- and VD-/Ca rats failed to improve, while control rats improved compared to initial performance. **(D)** There were no changes in forelimb grip strength, as measured by the force (g) required to break the rats grip. **(E-F)** Assessment of locomotor activity during a five minute recording using a force-plate actimeter revealed no changes in the **(E)** distance traveled or **(F)** number of rearing events. (*p<0.05 compared to control in same week, #p<0.05 compared to VD- in same week, ## p<0.05 compared to week 0 within the same group).

Figure 4.

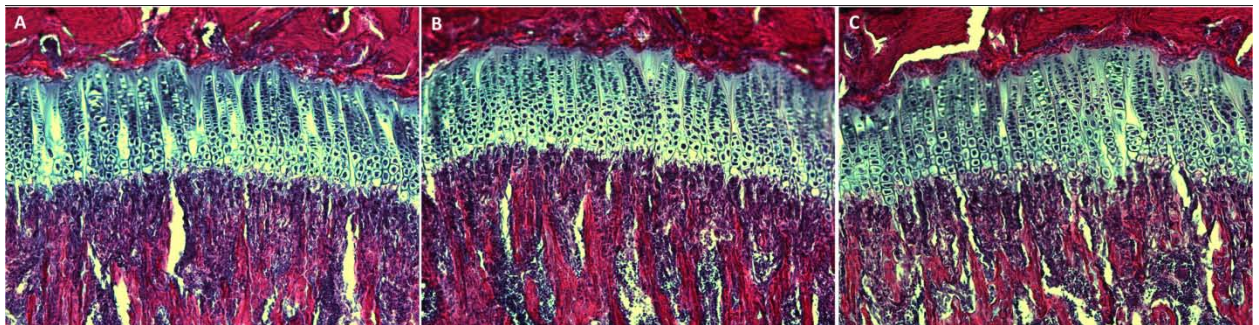


Figure 4. Representative images of tibial growth plates from rats after four weeks on (A) control, (B) VD-/Ca, or (C) VD- diets. Sagittal sections (20μm) of the growth plate stained with hematoxylin and eosin.

Figure 5.

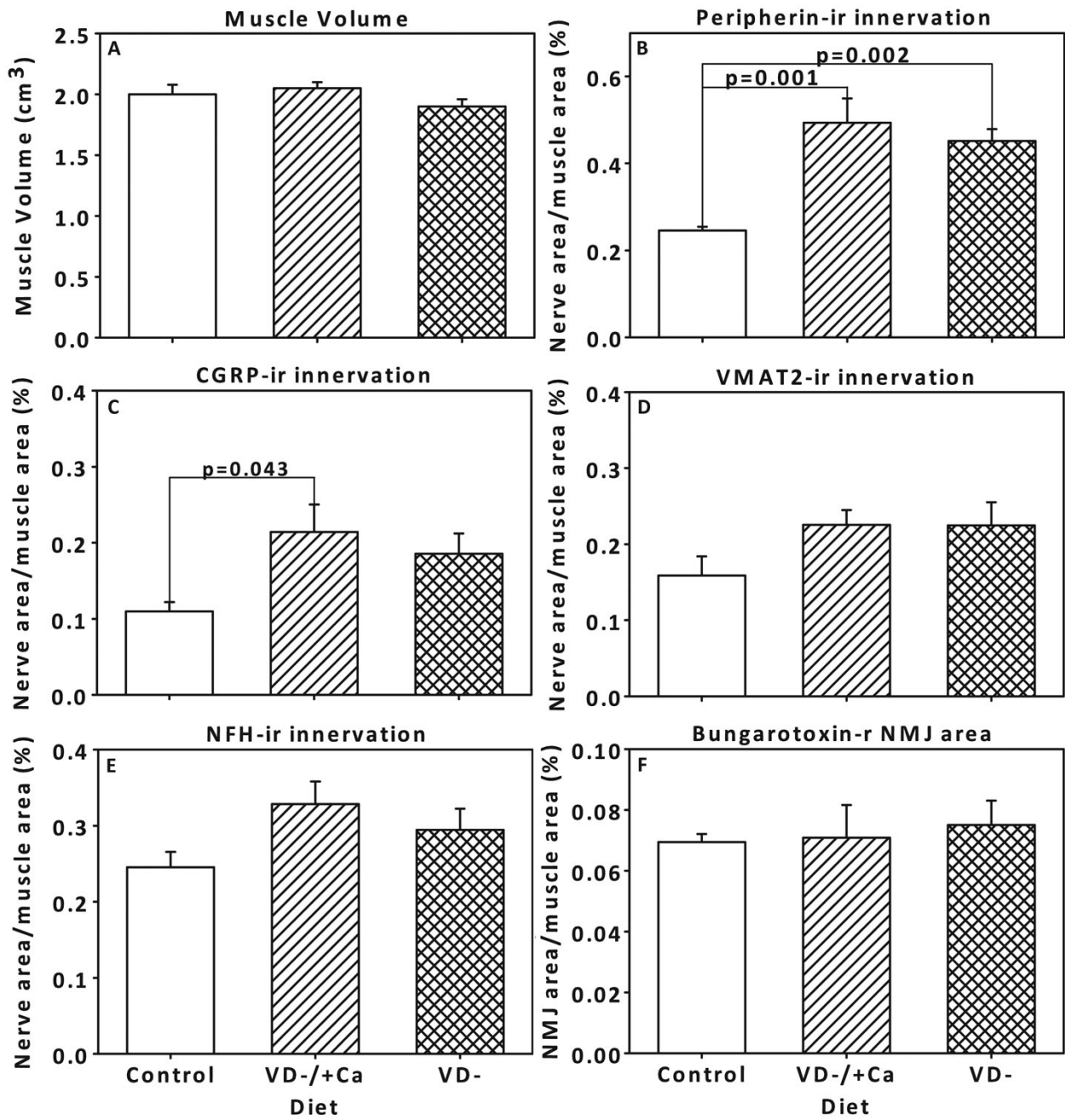


Figure 5. Comparisons of innervation densities of the lateral gastrocnemius muscles of rats after four weeks on control, VD-/ +Ca, or VD- diets. **(A)** There were no changes in the volumes of the calf muscles as measured by fluid displacement. **(A-E)** There were significant increases in the percentage of nerve area per field area of **(B)** peripherin-immunoreactive (ir) (c-fibers) and **(C)** CGRP-ir (sensory peptidergic/nociceptor) nerves, but not **(D)** VMAT2-ir (sympathetic) or **(E)** NFH-ir (large fiber) nerves. **(F)** There were also no changes in neuromuscular junction (NMJ) area as measured by bungarotoxin binding.

Figure 6.

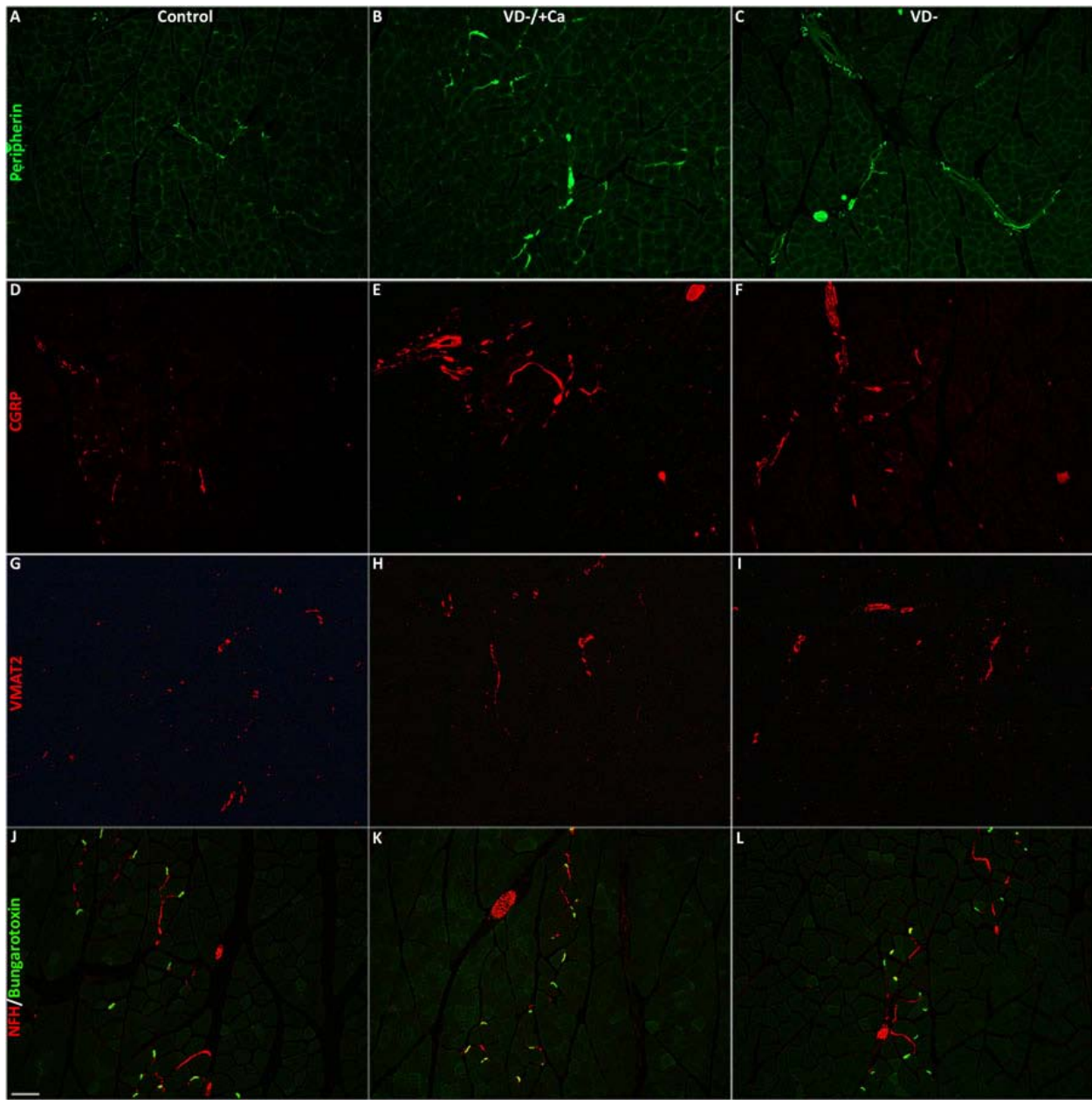


Figure 6. Representative images of the immunoreactive nerves in calf muscle. Transverse sections (20µm) of the lateral gastrocnemius of rats receiving **(A,D,G,J)** control, **(B,E,H,K)** VD-/ +Ca, or **(C,F,I,L)** VD- diets were immunofluorescently-labeled for **(A-C)** peripherin (green), **(D-F)** CGRP (red), **(G-I)** VMAT2 (red), or **(J-L)** NFH (red) and Bungarotoxin (green). (Scale 50µM)

Figure 7.

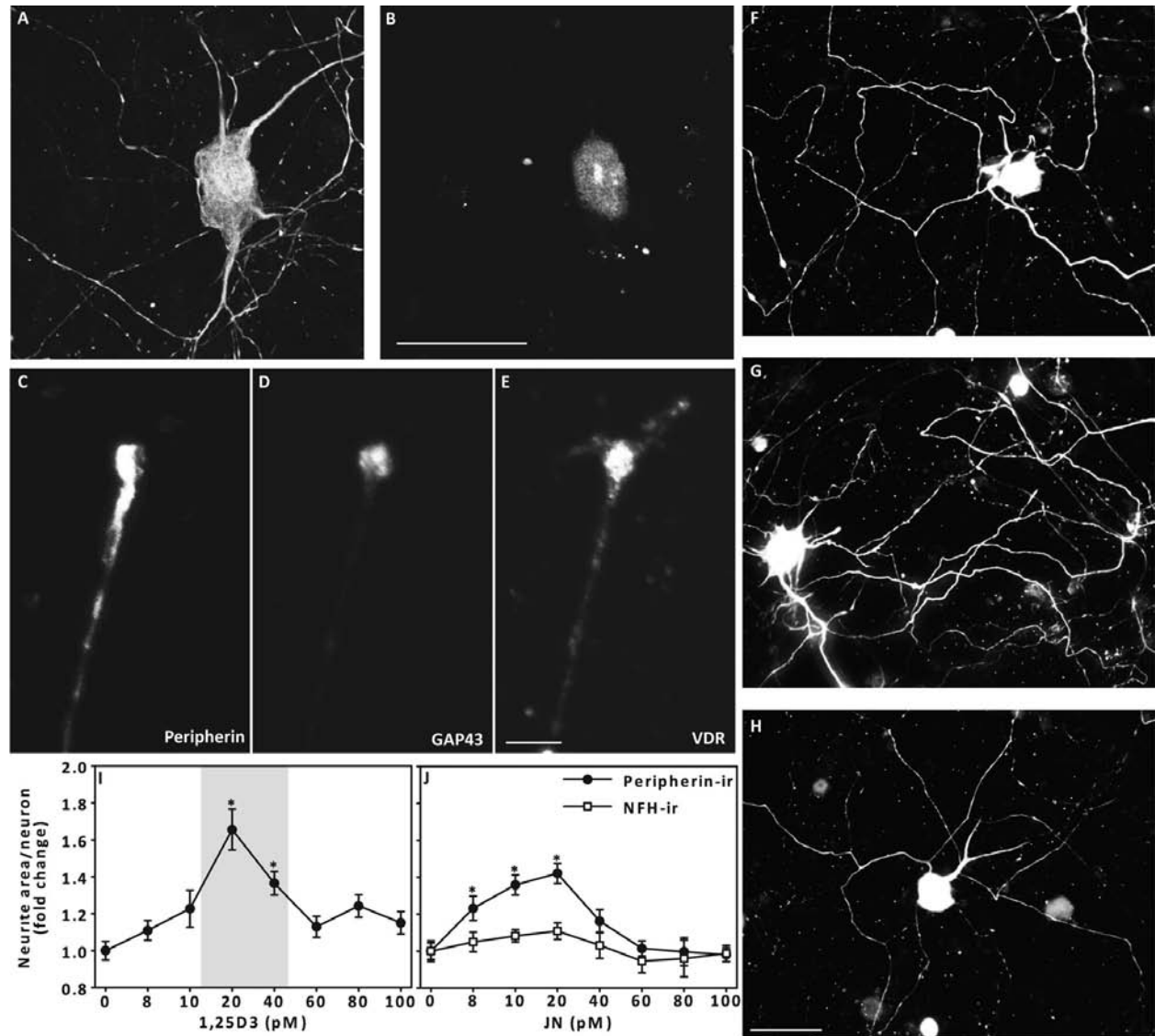


Figure 7. 1,25(OH)₂D₃ acts directly on c-fiber sensory nerves to regulate axonal growth. **(A-B)** Primary DRG cultures immunofluorescently stained for **(A)** peripherin and **(B)** vitamin D receptors (VDR), showing VDR is expressed by cultured sensory neurons, and localized to both the nucleus and the cytoplasm (Scale 50μm). **(C-E)** At a higher magnification it is possible to see that **(E)** VDR is localized to the tip of **(C)** peripherin-ir neurites, along with the growth cone marker, **(D)** growth associated protein 43 (GAP43) (Scale 5μm). **(F-H)** Representative images of neurons treated with **(F)** 0, **(G)** 20, or **(H)** 100pM 1,25(OH)₂D₃ (Scale 50μm). **(I)** Fold change in peripherin-ir neurite area per neuron at the indicated 1,25(OH)₂D₃ concentrations. Results reveal a biphasic curve with the peak at 20pM 1,25(OH)₂D₃. The shaded area corresponds to reported 1,25(OH)₂D₃ levels in patients with severe pain (Gloth, Lindsay et al. 1991). **(J)** DRG cultures treated with JN, a selective agonist for membrane/cytoplasm-localized rapid response VDRs, and stained for peripherin (●) or NFH (□). While there was no significant change in NFH-ir neurite area /neuron, there was a biphasic response to JN, with a peak at 20pM. (*p<0.05)

Figure 8.

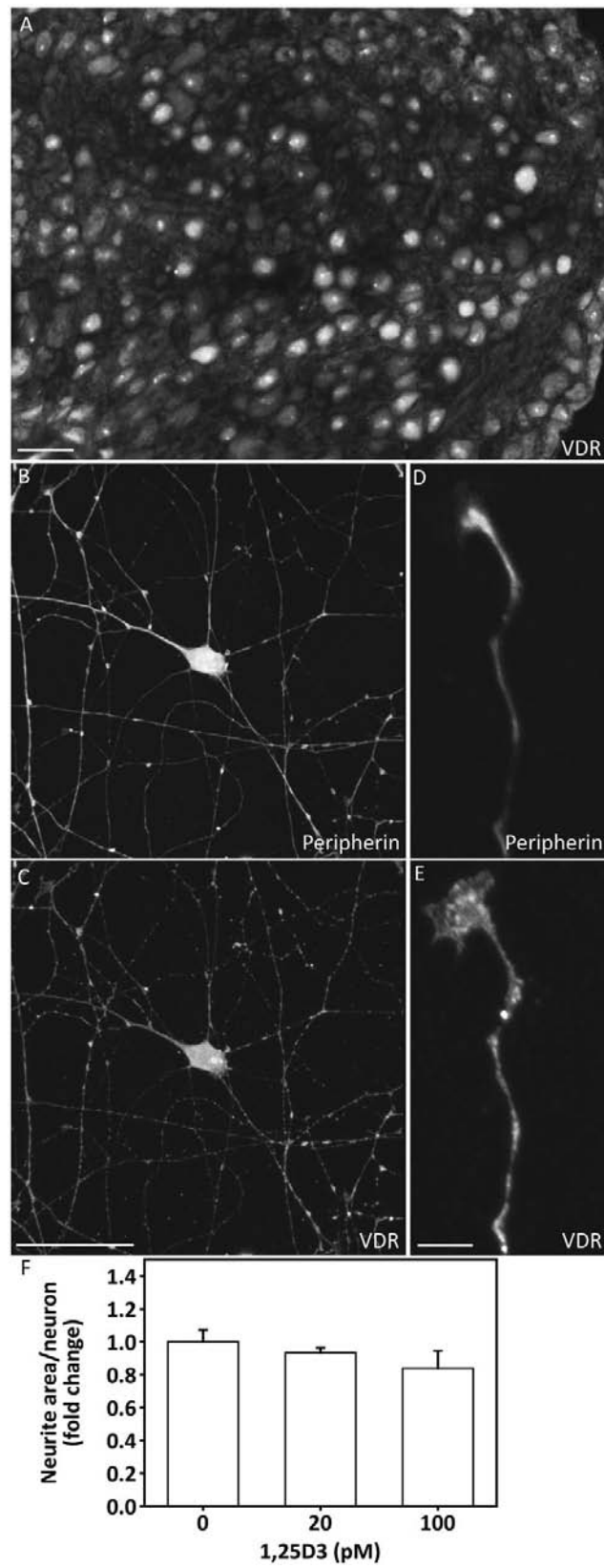


Figure 8. Sympathetic neurons express VDRs, but 1,25(OH)₂D₃ has no effect on neurite outgrowth. **(A)** Sympathetic neurons of superior cervical ganglia (SCG) were immunofluorescently labeled for VDR. VDR was localized to the nucleus and cytoplasm of selected neurons. **(B-E)** Primary SCG cultures immunofluorescently-stained for **(B&D)** peripherin and **(C&E)** VDR (Scale B&C 50μm, D&E 5μm). VDR was localized throughout the **(C)** cell body, **(E)** neurite, and growth cone. **(F)** Primary SCG cultures were grown for 48 hours with 0pM, 20pM, or 100pM 1,25(OH)₂D₃ and immunofluorescently labeled with peripherin. There were no significant changes in neurite area/neuron.

CHAPTER 4. GENERAL CONCLUSIONS AND DISCUSSION

While the evidence linking vitamin D deficiency and musculoskeletal pain has been accumulating, these are the first studies to show a direct causal relationship. In the rat model described here, vitamin D deficiency leads to deep tissue hypersensitivity, which occurred concurrently with muscle hyperinnervation by putative nociceptors. This hyperinnervation likely contributes to hypovitaminosis D-induced musculoskeletal pain and neither hypersensitivity nor hyperinnervation is related to serum calcium levels.

These are also the first studies to show direct actions of vitamin D metabolites on sensory neurons. Both vitamin D receptors and metabolic enzymes are found in c-fiber neurons, indicating the importance of tightly regulating vitamin D signaling in this cell population. At normal physiological concentrations, $1,25(\text{OH})_2\text{D}_3$ appears to directly act on membrane/cytoplasmic rapid response vitamin D receptors, possibly within the growth cone, to inhibit axonal sprouting from c-fiber sensory neurons. A vitamin D deficiency therefore reduces this inhibition, resulting in tissue hyperinnervation. These effects are cell-type specific, as hypovitaminosis D-induced axonal growth is not apparent with large fiber sensory neurons or sympathetic neurons either *in vivo* or in culture. Expression of both the ligand and cytoplasmic VDR is modulated by ovarian hormones, with the overall effect of reducing vitamin D signaling when ovarian hormones are suppressed. This in itself could lead to tissue hyperinnervation and hypersensitivity or perhaps exacerbate the effects of a vitamin D deficiency, accounting for the increased incidence of musculoskeletal pain in women with suppressed ovarian hormones.

Musculoskeletal Pain Management

With an estimated 27.5-36% of adolescents and 30-50% of adults suffering from chronic musculoskeletal pain (Bergman 2007; De Inocencio 2004), our current pain prevention and pain management strategies are clearly not effective. The most common drug therapies for chronic pain are NSAIDs (44%), weak opioids (i.e. tramadol, codeine) (23%), and acetaminophen (18%) (Breivik, Collett et al. 2006), all of which can have significant safety concerns. For example, 20% of patients taking

NSAIDs longer than two months will develop an ulcer and 1:1200 will die from gastroduodenal complications (Tramer, Moore et al. 2000); patients taking tramadol are at increased risk for developing seizures and life threatening serotonin syndrome (Sansone and Sansone 2009); and acetaminophen is reported to cause acute liver and renal failure if not taken properly (Graham, Scott et al. 2005). In addition to the potential side effects, in many cases these drugs are not efficient in managing pain. In fact only 36% of chronic pain patients report that their current therapies are adequate for managing pain (Breivik, Collett et al. 2006). Non-pharmacological pain management strategies on the other hand often have limited or short term effects. Massage, manipulation, or mobilization therapies may have short-term benefits, but there is no evidence that they have a positive long-term impact on chronic musculoskeletal pain (Airaksinen, Brox et al. 2006). There is also no evidence that hyperstimulation analgesia (i.e. acupuncture or transcutaneous electrical nerve stimulation) or relaxation techniques have lasting effects in studies analyzing chronic lower back pain (Airaksinen, Brox et al. 2006; Carroll and Seers 1998). Cognitive behavioral therapies, designed to change how patients perceive and react to their pain, have had positive results, but outcomes may be highly dependent on the social and psychological variables of the patient (McCracken and Turk 2002). Regular exercise may also help treat and prevent chronic pain, but patient adherence is low (Jordan, Holden et al. 2010). It therefore appears that neither current pharmacological nor alternative therapies for managing chronic musculoskeletal pain are sufficient.

This study is novel because it indicates that maintaining a healthy vitamin D status could prevent the development of some forms of muscle pain. The number of people suffering from hypovitaminosis D-induced musculoskeletal pain may be substantial, as up to 93% of patients with non-specific musculoskeletal pain are vitamin D deficient (Plotnikoff and Quigley 2003). While the model described here does not show whether vitamin D supplementation can reverse musculoskeletal pain once established, a number of clinical studies suggest that it can (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991). Proper vitamin D supplementation may therefore be a relatively easy, inexpensive, and safe way to treat and prevent non-specific musculoskeletal pain.

Tissue Hyperinnervation and increased sensitivity

Nociceptor hyperinnervation could increase peripheral input of pain signals to the spinal dorsal horn in a number of ways. It is known that actively growing axons show greater excitability than do quiescent axons (Janig, Grossmann et al. 2009). Neurons with more complex axonal geometries also show greater excitability (Janse, Peretz et al. 1999), perhaps because of greater spatial summation on the nociceptor itself. Similarly, more extensive axonal branching is likely to increase the number of nociceptors activated, resulting in greater spatial summation on postsynaptic terminals. It is also known that nociceptors can couple in the periphery (Meyer, Raja et al. 1985). In other words, the action potential of one neuron can initiate an action potential in adjacent neurons. If innervation density is increased, each neuron may come in contact with a larger population of neurons with which to couple, which could further increase the number of nociceptors activated per stimulus. Through any or all of these mechanisms, increased neurite outgrowth may enhance nociception.

Transition to Chronic Pain

Results from this study indicate that vitamin D deficiency causes peptidergic nociceptor hyperinnervation of the muscle, which could lower the nociceptive threshold and account for the observed deep tissue mechanical hypersensitivity. However, this hyperinnervation also has the potential to lead to chronic (unevoked) pain. In addition to their afferent actions, the peptidergic c-fibers, can antidromically release neuropeptides that promote neurogenic inflammation (Maggi 1995; Richardson and Vasko 2002). Once activated, the nerve endings release CGRP and tachykinins, which cause vasodilation, protein extravasation, mast cell degranulation, and infiltration and activation of immune cells, leading to tissue inflammation (Maggi 1995). This comes full circle, as deep tissue inflammation in turn further increases the production of CGRP and tachykinins (Ambalavanar, Moritani et al. 2006) and results in the release of cytokines, growth factors and other substances from the tissue, which sensitize nociceptors (Richardson and Vasko 2002). If peptidergic c-fibers are found at a high density in vitamin D deficient patients, neurogenic inflammation could be amplified as there are more nerve endings to release pro-inflammatory

substances. CGRP content itself may also be higher in the neurons of vitamin D deficient patients. $1,25(\text{OH})_2\text{D}$ is a well-established repressor of cAMP-induced CT/CGRP gene transcription and an inhibitor of CT and CGRP secretion from thyroid C-cells, thus with the removal of this repression, the loss of vitamin D may enhance CGRP expression and secretion and promote neurogenic inflammation (Baier, Grauer et al. 1994; Collignon, Laborie et al. 1992; Cote, Rogers et al. 1987; Lamari, Tahri et al. 1994; Lazaretti-Castro, Grauer et al. 1995; Lazaretti-Castro, Grauer et al. 1990; Naveh-Many, Raue et al. 1992; Naveh-Many and Silver 1988; Raue, Deutschle et al. 1984; Raue, Deutschle et al. 1983).

Furthermore, vitamin D deficient patients may be particularly susceptible to inflammation, as vitamin D metabolites act as inflammatory inhibitors (Baeke, Gysemans et al. 2010). Once sensitized by inflammation, the nociceptors may spontaneously discharge (Xiao and Bennett 2008). Prolonged firing of nociceptors, this can cause glutamate release in the spinal cord, which acts on NMDA receptors, leading to central sensitization (Bennett 2000). In addition, if CGRP levels are elevated in peptidergic nociceptors due to vitamin D deficiency, central sensitization may be further enhanced, as the central release of CGRP has been shown to potentiate the response of NMDA and non-NMDA receptors in the spinal dorsal horn, facilitating wind-up (Leem, Gwak et al. 2001). It is therefore possible that vitamin D deficient patients may transition from a state of deep tissue hypersensitivity to a chronic pain state through inflammation and central sensitization. At this point it is unclear whether vitamin D deficiency may play a role in establishing or maintaining central sensitization within the brain, but this possibility cannot be ruled out, as VDR is expressed in both neurons and glia throughout the brain (Baas, Prufer et al. 2000; Eyles, Smith et al. 2005; Glaser, Veenstra et al. 1999; Neveu, Naveilhan et al. 1994; Perez-Fernandez, Alonso et al. 1997; Prufer, Veenstra et al. 1999; Walbert, Jirikowski et al. 2001).

Gender differences and the role of sex hormones

Although both musculoskeletal pain and vitamin D deficiency are more common in women, vitamin D deficiency is also associated with musculoskeletal pain in men. Results from the European Male Ageing Study analyzing 3075 men ages 49-79, found that 8.6% of all men had chronic widespread

pain. 25.5% of men with chronic widespread pain had 25(OH)D levels below 15ng/ml (37.5nmol/L), compared to only 18.6% in men with no pain (McBeth, Pye et al. 2010), suggesting that vitamin D deficiency may cause musculoskeletal pain in men. However, chronic musculoskeletal pain in general is two times more prevalent in women than men (Bergman 2007). It seems to be associated with reduced ovarian hormones, as musculoskeletal pain is a common menopausal symptom and a major side-effect for estrogen lowering drugs (i.e. aromatase inhibitors, GnRH agonists) (Alexander, Dennerstein et al. 2007; Gaugris, Heaney et al. 2005; Khan, Reddy et al. 2010). This could simply be due to the increased incidence of vitamin D deficiency within this population; however, this study indicates that altered vitamin D signaling pathways within the nociceptors themselves may play a role. Sustained suppression of ovarian hormones results in suppression of both local ligand and receptor concentrations within peptidergic neurons, which might decrease vitamin D signaling and exacerbate the effects of a vitamin D deficiency. The clinical study by Plotnikoff and Quigley (Plotnikoff and Quigley 2003) supports this hypothesis. They examined 150 patients who sought clinical treatment for non-specific musculoskeletal pain. Reflecting the increased incidence of musculoskeletal pain in women, the study was comprised of 107 women and 43 men. Most of the women were over the age of 50 and therefore likely to be post-menopause. Almost all of the patients were vitamin D deficient (93%), but interestingly the women over the age of 50 had serum 25(OH)D levels that were significantly higher than either the women of childbearing age (<36 years old) or the men. Given our findings, this data may suggest that suppression of ovarian hormones in these postmenopausal women leads to reduced vitamin D signaling, causing musculoskeletal pain at earlier stages of a vitamin D deficiency. The reduced vitamin D signaling in putative nociceptors after hormone suppression may provide insight into another clinical situation. Patients taking aromatase inhibitors tend to be vitamin D deficient and have musculoskeletal pain (Khan, Reddy et al. 2010; Waltman, Ott et al. 2009). While vitamin D supplementation has been shown to be effective at reducing musculoskeletal pain in most vitamin D deficient patients, vitamin D supplementation only minimally reduced musculoskeletal pain in patients taking aromatase inhibitors, despite the fact that the supplementation increased serum levels of 25(OH)D (Khan, Reddy et al. 2010).

It is possible that aromatase inhibitor-induced hypoestrogenemia might severely hinder conversion of the therapeutic supplement to the active hormone, 1,25(OH)₂D, and thereby limit receptor-mediated signaling, rendering dietary supplementation less effective.

When vitamin D supplementation is not sufficient

The good news is that many people with musculoskeletal pain may find relief with vitamin D supplementation, which when done correctly has no side effects (Heaney 2008). In fact, a number of studies have shown this to be the case (de Torrente de la Jara, Pecoud et al. 2006; Glerup and Eriksen 1999; Gloth, Lindsay et al. 1991). However, understanding the mechanism may prove important for some populations who may not improve with simple vitamin D supplementation. Patients taking estrogen lowering drugs as described above may be one population. In addition, patients on dialysis, who have a compromised ability to convert 25(OH)D to 1,25(OH)₂D and who often suffer from musculoskeletal pain (Golan, Haggagi et al. 2009), may not benefit from vitamin D supplementation either, because they cannot produce adequate levels of active ligand. One option is Vitamin D receptor activators (i.e. calcitriol); however, these carry a higher risk than traditional vitamin D supplements, because of their ability to promote hypercalcemia. Alternatively, based on our findings that vitamin D deficiency causes an increase in the density of peptidergic c-fibers within the muscle, it is possible that therapies that desensitize these peripheral nerve endings (i.e. TRPV1 antagonists) may prove beneficial for musculoskeletal pain.

Dietary calcium and hypovitaminosis D-induced musculoskeletal pain

Some of the effects of a vitamin D deficiency are not due to the direct actions of this hormone, but rather due to secondary hypocalcemia (i.e. reduced fertility and increased vascular muscle contractility) (Johnson and DeLuca 2002; Uhland, Kwiecinski et al. 1992; Weishaar and Simpson 1987). However, it appears that hypovitaminosis D-induced muscle pain is not due to secondary hypocalcemia.

In fact, the results from this study suggest that high dietary calcium concentrations in vitamin D deficiency may actually exacerbate this condition. It is interesting to note that all patients with severe pain in the study by Gloth et. al. had low 1,25(OH)₂D serum concentrations, normal serum calcium concentrations, and most were taking a calcium supplement (Gloth, Lindsay et al. 1991). Dietary calcium concentrations are important to consider, because they may affect 1,25(OH)₂D concentrations.

1,25(OH)₂D does not always decrease in a vitamin D deficiency, and in fact when serum 25D levels drop below 40nM, 1,25(OH)₂D levels often increase (Need, Horowitz et al. 2000). This is because as serum Ca concentrations drop in a vitamin D deficiency, parathyroid hormone levels increase, which increases expression of CYP27B1, the enzyme responsible for converting 25(OH)D to 1,25(OH)₂D. It is possible that high dietary calcium may prevent the drop in serum calcium that leads to increased PTH levels, thereby preventing a spike in 1,25(OH)₂D levels. This appears to be the case in rats, because increased dietary calcium inhibits expression of CYP27B1, abrogates the 1,25(OH)₂D spike, and accelerates the decrease of 1,25(OH)₂D in a vitamin D deficiency (Anderson, Lee et al. 2010; Mallon, Boris et al. 1981). If high dietary calcium reduces the levels of active hormone, this could be the reason why increasing dietary calcium accelerated the onset of deep tissue hypersensitivity in this study. In view of how common dietary calcium supplementation is (43% of population use Ca supplements) (Bailey, Dodd et al. 2010), it may be an import aspect for a doctor to consider when suspecting hypovitaminosis D-induced musculoskeletal pain.

Beyond Pain

It is unlikely that nociceptor-sprouting in response to vitamin D deficiency evolved for the sole purpose of causing pain, so it is interesting to consider the evolutionary benefits of this process. One potential benefit could be increased regulation of blood flow to skeletal muscle. Vitamin D deficiency is already known to have profound effects on the cardiovascular system. As vitamin D levels drop, blood pressure rises (Scragg, Sowers et al. 2007). This alone could increase tissue blood flow in vitamin D deficiency. This may be important for individuals living at higher latitudes, who experience dramatic

seasonal variations in temperature, have low serum 25(OH)D during the winter months (Holick 2005), and are known to have elevated blood pressures in the winter (Woodhouse, Khaw et al. 1993). If this drop in vitamin D levels results in increased blood flow to peripheral tissues, it could be important for maintaining heat in the extremities during cold winters. In addition, the increased c-fiber innervation of skeletal muscle could also promote thermogenesis. Shivering thermogenesis is important for maintaining basal body temperatures in cold environments and involves the involuntary, rhythmic contractions of skeletal muscles. Shivering requires a dramatic increase in oxygen consumption (~5 times the resting metabolic rate). An increase in peptidergic c-fibers in skeletal muscle, could locally increase oxygen delivery, by priming the blood vessels in the muscle for vasodilation. Upon activation of these nerves, peptidergic nociceptors antidromically release CGRP, which promotes vasodilation. For example, electrical stimulation of the saphenous nerve results in muscle arteriolar dilation and increased blood flow to the gracilis muscle, which can be prevented by the CGRP receptor antagonist, CGRP₈₋₃₇ (Loaiza, Yamaguchi et al. 2002). The hypovitaminosis D-induced increases in the innervation of muscle by CGRP-containing nerves may therefore play a role in further increasing blood flow to the skeletal muscle, providing thermogenic advantages in colder climates. In fact, it is known that individuals from higher latitude cold weather climates (e.g. Alaskans), who are known to have lower vitamin D levels (Holick 2005) in the winter, are able to maintain higher temperatures in their extremities when exposed to cold than individuals from warmer climates, making them less susceptible to frost-bite (Miller and Irving 1962).

Potential Mechanisms

The results from this study indicate that 1,25(OH)₂D₃ is capable of acting directly on sensory c-fibers to regulate neurite outgrowth. 1,25(OH)₂D₃ likely acts through membrane/cytoplasmic VDRs, which typically initiate rapid response signaling cascades. The rapid response specific agonist, JN, has similar dose dependent effects on neurite growth as 1,25(OH)₂D₃. These rapid response cascades could be initiated locally within the growth cone, as VDR appears to be enriched there. (Mizwicki and Norman

2009). The rapid responses known to be mediated by $1,25(\text{OH})_2\text{D}$ are likely cell type specific, but include PKC, MAPK, PLC, PLA_2 , Src, and Raf activation, opening Ca^{2+} and Cl^- channels, and sphingomyelin hydrolysis (Mizwicki and Norman 2009). Any or all of these downstream signals could play a role in neurite outgrowth. One of the best characterized VDR rapid responses is regulation of the flux of ions such as calcium. Like VDR activation, calcium modulation has a biphasic curve on neurite outgrowth (Gomez and Zheng 2006). In other cell types, membrane VDR regulates intracellular calcium concentrations through modulation of L-type calcium channels (Zanello and Norman 2004). This is interesting, because L-type voltage gated channels are the primary source for inward calcium flux in c-fiber neurons (Scroggs and Fox 1992) and they are known regulators of sensory neurite outgrowth (Archer, Doherty et al. 1999). Therefore, while serum calcium concentrations were not a factor in the development of muscle pain, $1,25(\text{OH})_2\text{D}$ may regulate intracellular calcium concentrations within the axon.

Conclusion

Collectively, these results show that vitamin D deficiency does cause deep tissue hypersensitivity in rats. $1,25(\text{OH})_2\text{D}_3$ acts directly on membrane/cytoplasmic rapid response VDRs to regulate axonal growth from a sensory subtype considered to be nociceptive. During a vitamin D deficiency, the reduced signaling from these rapid response VDR receptors leads to sensory hyperinnervation of muscle, which may contribute to the development of deep tissue hypersensitivity in rats. Suppression of ovarian hormones may further promote hypovitaminosis D-induced musculoskeletal pain by reducing vitamin D signaling within nociceptors. This may contribute to the high prevalence of musculoskeletal pain in women with low levels of ovarian hormones. Preventing the development of non-specific musculoskeletal pain may be as simple as supplementing the diet with vitamin D.

REFERENCES

- Abusdal UG, Hagen KB, et al. (1997). [Self-reported chronic muscle pain among women in Oslo]. *Tidsskr Nor Laegeforen* 117(11): 1606-1610.
- Airaksinen O, Brox JJ, et al. (2006). Chapter 4. European guidelines for the management of chronic nonspecific low back pain. *Eur Spine J* 15 Suppl 2: S192-300.
- Alexander JL, Dennerstein L, et al. (2007). Arthralgias, bodily aches and pains and somatic complaints in midlife women: etiology, pathophysiology and differential diagnosis. *Expert Rev Neurother* 7(11 Suppl): S15-26.
- Alfredson H, Ohberg L, et al. (2003). Is vasculo-neural ingrowth the cause of pain in chronic Achilles tendinosis? An investigation using ultrasonography and colour Doppler, immunohistochemistry, and diagnostic injections. *Knee Surg Sports Traumatol Arthrosc* 11(5): 334-338.
- Ambalavanar R, Moritani M, et al. (2006). Deep tissue inflammation upregulates neuropeptides and evokes nociceptive behaviors which are modulated by a neuropeptide antagonist. *Pain* 120(1-2): 53-68.
- Ambalavanar R and Morris R (1993). An ultrastructural study of the binding of an alpha-D-galactose specific lectin from *Griffonia simplicifolia* to trigeminal ganglion neurons and the trigeminal nucleus caudalis in the rat. *Neuroscience* 52(3): 699-709.
- Anderson PH, Lee AM, et al. (2010). The effect of dietary calcium on 1,25(OH)₂D₃ synthesis and sparing of serum 25(OH)D₃ levels. *J Steroid Biochem Mol Biol* 121(1-2): 288-292.
- Andersson H, Ejlertsson G, et al. (1998). Widespread musculoskeletal chronic pain associated with smoking. An epidemiological study in a general rural population. *Scand J Rehabil Med* 30(3): 185-191.
- Andersson HI, Ejlertsson G, et al. (1993). Chronic pain in a geographically defined general population: studies of differences in age, gender, social class, and pain localization. *Clin J Pain* 9(3): 174-182.
- Andersson HI, Ejlertsson G, et al. (1999). Musculoskeletal chronic pain in general practice. Studies of health care utilisation in comparison with pain prevalence. *Scand J Prim Health Care* 17(2): 87-92.
- Annweiler C, Montero-Odasso M, et al. (2010). Fall prevention and vitamin D in the elderly: an overview of the key role of the non-bone effects. *J Neuroeng Rehabil* 7: 50.
- Archer FR, Doherty P, et al. (1999). CAMs and FGF cause a local submembrane calcium signal promoting axon outgrowth without a rise in bulk calcium concentration. *Eur J Neurosci* 11(10): 3565-3573.

- Armas LA, Dowell S, et al. (2007). Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: The effect of UVB dose and skin color. *J Am Acad Dermatol*.
- Arunabh S, Pollack S, et al. (2003). Body fat content and 25-hydroxyvitamin D levels in healthy women. *J Clin Endocrinol Metab* 88(1): 157-161.
- Awareness TCFD (2006). The 2006 CDA Long-Term Disability Claims Review. 1-3.
- Baas D, Prufer K, et al. (2000). Rat oligodendrocytes express the vitamin D(3) receptor and respond to 1,25-dihydroxyvitamin D(3). *Glia* 31(1): 59-68.
- Baeke F, Gysemans C, et al. (2010). Vitamin D insufficiency: implications for the immune system. *Pediatr Nephrol* 25(9): 1597-1606.
- Baier R, Grauer A, et al. (1994). Differential effects of 1,25-dihydroxyvitamin D3 on cell proliferation and calcitonin gene expression. *Endocrinology* 135(5): 2006-2011.
- Bailey RL, Dodd KW, et al. (2010). Estimation of total usual calcium and vitamin D intakes in the United States. *J Nutr* 140(4): 817-822.
- Becker JB, Arnold AP, et al. (2005). Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* 146(4): 1650-1673.
- Bell NH, Godsen RN, et al. (1988). The effects of muscle-building exercise on vitamin D and mineral metabolism. *J Bone Miner Res* 3(4): 369-373.
- Bennett DL, Michael GJ, et al. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 18(8): 3059-3072.
- Bennett GJ (2000). Update on the neurophysiology of pain transmission and modulation: focus on the NMDA-receptor. *J Pain Symptom Manage* 19(1 Suppl): S2-6.
- Benson J, Wilson A, et al. (2006). Muscle pain as an indicator of vitamin D deficiency in an urban Australian Aboriginal population. *Med J Aust* 185(2): 76-77.
- Bergman S (2007). Public health perspective--how to improve the musculoskeletal health of the population. *Best Pract Res Clin Rheumatol* 21(1): 191-204.
- Bergman S, Herrstrom P, et al. (2001). Chronic musculoskeletal pain, prevalence rates, and sociodemographic associations in a Swedish population study. *J Rheumatol* 28(6): 1369-1377.
- Berkley KJ, Robbins A, et al. (1993). Functional differences between afferent fibers in the hypogastric and pelvic nerves innervating female reproductive organs in the rat. *J Neurophysiol* 69(2): 533-544.

- Birklein F and Schmelz M (2008). Neuropeptides, neurogenic inflammation and complex regional pain syndrome (CRPS). *Neurosci Lett* 437(3): 199-202.
- Bischof MG, Heinze G, et al. (2006). Vitamin D status and its relation to age and body mass index. *Horm Res* 66(5): 211-215.
- Bischoff-Ferrari HA, Dawson-Hughes B, et al. (2004). Effect of Vitamin D on falls: a meta-analysis. *Jama* 291(16): 1999-2006.
- Blacklock AD, Johnson MS, et al. (2005). Estrogen increases sensory nociceptor neuritogenesis in vitro by a direct, nerve growth factor-independent mechanism. *Eur J Neurosci* 21(9): 2320-2328.
- Block SR (2004). Vitamin D deficiency is not associated with nonspecific musculoskeletal pain syndromes including fibromyalgia. *Mayo Clin Proc* 79(12): 1585-1586; author reply 1586-1587.
- Bohm-Starke N, Hilliges M, et al. (1999). Neurochemical characterization of the vestibular nerves in women with vulvar vestibulitis syndrome. *Gynecol Obstet Invest* 48(4): 270.
- Bolland MJ, Grey AB, et al. (2007). Age-, gender-, and weight-related effects on levels of 25-hydroxyvitamin D are not mediated by vitamin D binding protein. *Clin Endocrinol (Oxf)* 67(2): 259-264.
- Brage S and Bjerkedal T (1996). Musculoskeletal pain and smoking in Norway. *J Epidemiol Community Health* 50(2): 166-169.
- Breivik H, Collett B, et al. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur J Pain* 10(4): 287-333.
- Carroll D and Seers K (1998). Relaxation for the relief of chronic pain: a systematic review. *J Adv Nurs* 27(3): 476-487.
- Casellini CM and Vinik AI (2007). Clinical manifestations and current treatment options for diabetic neuropathies. *Endocr Pract* 13(5): 550-566.
- Cauley JA, Gutai JP, et al. (1989). The epidemiology of serum sex hormones in postmenopausal women. *Am J Epidemiol* 129(6): 1120-1131.
- Cekic M, Sayeed I, et al. (2009). Combination treatment with progesterone and vitamin D hormone may be more effective than monotherapy for nervous system injury and disease. *Front Neuroendocrinol* 30(2): 158-172.

- Chan JR, Rodriguez-Waitkus PM, et al. (2000). Progesterone synthesized by Schwann cells during myelin formation regulates neuronal gene expression. *Mol Biol Cell* 11(7): 2283-2295.
- Chapuy MC, Preziosi P, et al. (1997). Prevalence of vitamin D insufficiency in an adult normal population. *Osteoporos Int* 7(5): 439-443.
- Clarke GL, Bhattacharjee A, et al. (2010). Beta-adrenoceptor blockers increase cardiac sympathetic innervation by inhibiting autoreceptor suppression of axon growth. *J Neurosci* 30(37): 12446-12454.
- Clemens TL, Adams JS, et al. (1982). Increased skin pigment reduces the capacity of skin to synthesise vitamin D3. *Lancet* 1(8263): 74-76.
- Collignon H, Laborie C, et al. (1992). Effects of dexamethasone, calcium and 1,25-dihydroxycholecalciferol on calcitonin and calcitonin gene-related peptide mRNA levels from the CA-77 C cell line. *Thyroid* 2(4): 361-365.
- Cornet A, Baudet C, et al. (1998). 1,25-Dihydroxyvitamin D3 regulates the expression of VDR and NGF gene in Schwann cells in vitro. *J Neurosci Res* 53(6): 742-746.
- Cote GJ, Rogers DG, et al. (1987). The effect of 1,25-dihydroxyvitamin D3 treatment on calcitonin and calcitonin gene-related peptide mRNA levels in cultured human thyroid C-cells. *Biochem Biophys Res Commun* 149(1): 239-243.
- Craft RM (2007). Modulation of pain by estrogens. *Pain* 132 Suppl 1: S3-12.
- Croft P, Rigby AS, et al. (1993). The prevalence of chronic widespread pain in the general population. *J Rheumatol* 20(4): 710-713.
- Cummings SR, Black DM, et al. (1993). Bone density at various sites for prediction of hip fractures. The Study of Osteoporotic Fractures Research Group. *Lancet* 341(8837): 72-75.
- Dawson-Hughes B, Harris SS, et al. (1997). Plasma calcidiol, season, and serum parathyroid hormone concentrations in healthy elderly men and women. *Am J Clin Nutr* 65(1): 67-71.
- Dawson-Hughes B, Heaney RP, et al. (2005). Estimates of optimal vitamin D status. *Osteoporos Int* 16(7): 713-716.
- De Inocencio J (2004). Epidemiology of musculoskeletal pain in primary care. *Arch Dis Child* 89(5): 431-434.
- De Rezende Pena C, Grillo LP, et al. (2010). Evaluation of 25-hydroxyvitamin d serum levels in patients with fibromyalgia. *J Clin Rheumatol* 16(8): 365-369.

- De Torrente De La Jara G, Pecoud A, et al. (2006). Female asylum seekers with musculoskeletal pain: the importance of diagnosis and treatment of hypovitaminosis D. *BMC Fam Pract* 7: 4.
- Di Sebastiano P, Fink T, et al. (1995). Changes of protein gene product 9.5 (PGP 9.5) immunoreactive nerves in inflamed appendix. *Dig Dis Sci* 40(2): 366-372.
- Di Sebastiano P, Fink T, et al. (1997). Immune cell infiltration and growth-associated protein 43 expression correlate with pain in chronic pancreatitis. *Gastroenterology* 112(5): 1648-1655.
- Diesel B, Radermacher J, et al. (2005). Vitamin D(3) metabolism in human glioblastoma multiforme: functionality of CYP27B1 splice variants, metabolism of calcidiol, and effect of calcitriol. *Clin Cancer Res* 11(15): 5370-5380.
- Dugan SA, Powell LH, et al. (2006). Musculoskeletal pain and menopausal status. *Clin J Pain* 22(4): 325-331.
- Duncan WE, Glass AR, et al. (1991). Estrogen regulation of the nuclear 1,25-dihydroxyvitamin D3 receptor in rat liver and kidney. *Endocrinology* 129(5): 2318-2324.
- Duque G, El Abdaimi K, et al. (2002). Estrogens (E2) regulate expression and response of 1,25-dihydroxyvitamin D3 receptors in bone cells: changes with aging and hormone deprivation. *Biochem Biophys Res Commun* 299(3): 446-454.
- Edwards RR, Doleys DM, et al. (2001). Ethnic differences in pain tolerance: clinical implications in a chronic pain population. *Psychosom Med* 63(2): 316-323.
- Escaleira MT, Sonohara S, et al. (1993). Sex steroids induced up-regulation of 1,25-(OH)₂ vitamin D3 receptors in T 47D breast cancer cells. *J Steroid Biochem Mol Biol* 45(4): 257-263.
- Eyles D, Brown J, et al. (2003). Vitamin D3 and brain development. *Neuroscience* 118(3): 641-653.
- Eyles DW, Smith S, et al. (2005). Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. *J Chem Neuroanat* 29(1): 21-30.
- Faulkner KA, Cauley JA, et al. (2006). Higher 1,25-dihydroxyvitamin D3 concentrations associated with lower fall rates in older community-dwelling women. *Osteoporos Int* 17(9): 1318-1328.
- Felson DT and Cummings SR (2005). Aromatase inhibitors and the syndrome of arthralgias with estrogen deprivation. *Arthritis Rheum* 52(9): 2594-2598.

- Fink T, Di Sebastiano P, et al. (1994). Growth-associated protein-43 and protein gene-product 9.5 innervation in human pancreas: changes in chronic pancreatitis. *Neuroscience* 63(1): 249-266.
- Ford L, Graham V, et al. (2006). Vitamin D concentrations in an UK inner-city multicultural outpatient population. *Ann Clin Biochem* 43(Pt 6): 468-473.
- Fowler SC, Birkestrand BR, et al. (2001). A force-plate actometer for quantitating rodent behaviors: illustrative data on locomotion, rotation, spatial patterning, stereotypies, and tremor. *J Neurosci Methods* 107(1-2): 107-124.
- Freeman ME (1994). The Neuroendocrine Control of the Ovarian Cycle of the Rat. The Physiology of Reproduction. J. D. N. E. Knobil. New York, Raven Press: 613-658.
- Friedman AJ, Juneau-Norcross M, et al. (1993). Adverse effects of leuprolide acetate depot treatment. *Fertil Steril* 59(2): 448-450.
- Gangula PR, Lanlua P, et al. (2000). Regulation of calcitonin gene-related peptide expression in dorsal root ganglia of rats by female sex steroid hormones. *Biol Reprod* 62(4): 1033-1039.
- Gansky SA and Plesh O (2007). Widespread pain and fibromyalgia in a biracial cohort of young women. *J Rheumatol* 34(4): 810-817.
- Garreau JR, Delamelena T, et al. (2006). Side effects of aromatase inhibitors versus tamoxifen: the patients' perspective. *Am J Surg* 192(4): 496-498.
- Gaugris S, Heaney RP, et al. (2005). Vitamin D inadequacy among post-menopausal women: a systematic review. *QJM* 98(9): 667-676.
- Gilad LA, Bresler T, et al. (2005). Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. *J Endocrinol* 185(3): 577-592.
- Glaser SD, Veenstra TD, et al. (1999). Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat olfactory system. *Cell Mol Neurobiol* 19(5): 613-624.
- Glerup H and Eriksen EF (1999). Acroparaesthesia--a typical finding in vitamin D deficiency. *Rheumatology (Oxford)* 38(5): 482.
- Glerup H, Mikkelsen K, et al. (2000). Hypovitaminosis D myopathy without biochemical signs of osteomalacic bone involvement. *Calcif Tissue Int* 66(6): 419-424.
- Gloth FM, 3rd, Lindsay JM, et al. (1991). Can vitamin D deficiency produce an unusual pain syndrome? *Arch Intern Med* 151(8): 1662-1664.

- Golan E, Haggiag I, et al. (2009). Calcium, parathyroid hormone, and vitamin D: major determinants of chronic pain in hemodialysis patients. *Clin J Am Soc Nephrol* 4(8): 1374-1380.
- Goldstein ME, House SB, et al. (1991). NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. *J Neurosci Res* 30(1): 92-104.
- Gomez TM and Zheng JQ (2006). The molecular basis for calcium-dependent axon pathfinding. *Nat Rev Neurosci* 7(2): 115-125.
- Gonzalez Pardo V, Boland R, et al. (2008). Vitamin D receptor levels and binding are reduced in aged rat intestinal subcellular fractions. *Biogerontology* 9(2): 109-118.
- Graham GG, Scott KF, et al. (2005). Tolerability of paracetamol. *Drug Saf* 28(3): 227-240.
- Gray TK, Mcadoo T, et al. (1982). Fluctuation of serum concentration of 1,25-dihydroxyvitamin D3 during the menstrual cycle. *Am J Obstet Gynecol* 144(8): 880-884.
- Greendale GA, Reboussin BA, et al. (1998). Symptom relief and side effects of postmenopausal hormones: results from the Postmenopausal Estrogen/Progestin Interventions Trial. *Obstet Gynecol* 92(6): 982-988.
- Headley DB, Suhan NM, et al. (2007). Different subcellular distributions of the vesicular monoamine transporter, VMAT2, in subclasses of sympathetic neurons. *Brain Res* 1129(1): 156-160.
- Heaney RP (2008). Vitamin D: criteria for safety and efficacy. *Nutr Rev* 66(10 Suppl 2): S178-181.
- Heaney RP, Dowell MS, et al. (2003). Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. *J Am Coll Nutr* 22(2): 142-146.
- Heidari B, Shirvani JS, et al. (2010). Association between nonspecific skeletal pain and vitamin D deficiency. *Int J Rheum Dis* 13(4): 340-346.
- Ho SC, Chan SG, et al. (1999). Menopausal symptoms and symptom clustering in Chinese women. *Maturitas* 33(3): 219-227.
- Holick MF (2005). Photobiology of Vitamin D. Vitamin D. D. Feldman, F. H. Glorieux and J. W. Pike. Amsterdam ; Boston, Elsevier Academic Press. **1**: 37-43.
- Holick MF (2005). Photobiology of Vitamin D. Vitamin D. D. Feldman, F. H. Glorieux and J. W. Pike. Amsterdam ; Boston, Elsevier Academic Press. **1**: 37-45.
- Holick MF (2009). Vitamin D status: measurement, interpretation, and clinical application. *Ann Epidemiol* 19(2): 73-78.

- Holick MF and Chen TC (2008). Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr* 87(4): 1080S-1086S.
- Holick MF, Matsuoka LY, et al. (1989). Age, vitamin D, and solar ultraviolet. *Lancet* 2(8671): 1104-1105.
- Hollis BW (2005). Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J Nutr* 135(2): 317-322.
- Horst RL, Reinhardt TA, et al. (2005). Vitamin D Metabolism. Vitamin D. D. Feldman, F. H. Glorieux and J. W. Pike. Amsterdam ; Boston, Elsevier Academic Press.
- Huhtakangas JA, Olivera CJ, et al. (2004). The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)₂-vitamin D₃ in vivo and in vitro. *Mol Endocrinol* 18(11): 2660-2671.
- Hunt IM, Silman AJ, et al. (1999). The prevalence and associated features of chronic widespread pain in the community using the 'Manchester' definition of chronic widespread pain. *Rheumatology (Oxford)* 38(3): 275-279.
- Institute of Medicine FaNB (2010). Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC.
- Janig W, Grossmann L, et al. (2009). Mechano- and thermosensitivity of regenerating cutaneous afferent nerve fibers. *Exp Brain Res* 196(1): 101-114.
- Janse C, Peretz B, et al. (1999). Excitability and branching of neuroendocrine cells during reproductive senescence. *Neurobiol Aging* 20(6): 675-683.
- Johnson JA, Grande JP, et al. (1996). 1,25-Dihydroxyvitamin D(3) receptors in developing dorsal root ganglia of fetal rats. *Brain Res Dev Brain Res* 92(1): 120-124.
- Johnson LE and Deluca HF (2002). Reproductive defects are corrected in vitamin d-deficient female rats fed a high calcium, phosphorus and lactose diet. *J Nutr* 132(8): 2270-2273.
- Jones G (2007). Expanding role for vitamin D in chronic kidney disease: importance of blood 25-OH-D levels and extra-renal 1alpha-hydroxylase in the classical and nonclassical actions of 1alpha,25-dihydroxyvitamin D(3). *Semin Dial* 20(4): 316-324.
- Jones G (2008). Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr* 88(2): 582S-586S.
- Jones KD, Adams D, et al. (2006). A comprehensive review of 46 exercise treatment studies in fibromyalgia (1988-2005). *Health Qual Life Outcomes* 4: 67.

- Jordan JL, Holden MA, et al. (2010). Interventions to improve adherence to exercise for chronic musculoskeletal pain in adults. *Cochrane Database Syst Rev*(1): CD005956.
- Khan QJ, Reddy PS, et al. (2009). Effect of vitamin D supplementation on serum 25-hydroxy vitamin D levels, joint pain, and fatigue in women starting adjuvant letrozole treatment for breast cancer. *Breast Cancer Res Treat*.
- Khan QJ, Reddy PS, et al. (2010). Effect of vitamin D supplementation on serum 25-hydroxy vitamin D levels, joint pain, and fatigue in women starting adjuvant letrozole treatment for breast cancer. *Breast Cancer Res Treat* 119(1): 111-118.
- Klausen T, Breum L, et al. (1993). Plasma levels of parathyroid hormone, vitamin D, calcitonin, and calcium in association with endurance exercise. *Calcif Tissue Int* 52(3): 205-208.
- Lamari Y, Tahri EH, et al. (1994). Steroid hormones and retinoic acid interact in the regulation of calcitonin and calcitonin gene-related peptide secretion and messenger ribonucleic acid levels in CA-77 C cells. *Cell Mol Biol (Noisy-le-grand)* 40(4): 541-550.
- Lawson SN (1992). Morphological and biochemical cell types of sensory neurons. Sensory neurons: diversity, development, and plasticity. S. A. Scott. New York, Oxford Press: 27-59.
- Lazaretti-Castro M, Grauer A, et al. (1995). 1,25-Dihydroxyvitamin D3 stimulates growth and inhibits calcitonin secretion in a human C cell carcinoma cell line. *Braz J Med Biol Res* 28(9): 1013-1018.
- Lazaretti-Castro M, Grauer A, et al. (1990). 1,25-Dihydroxyvitamin D3 suppresses dexamethasone effects on calcitonin secretion. *Mol Cell Endocrinol* 71(3): R13-18.
- Lechner D, Bajna E, et al. (2006). Genistein and 17beta-estradiol, but not equol, regulate vitamin D synthesis in human colon and breast cancer cells. *Anticancer Res* 26(4A): 2597-2603.
- Leem JW, Gwak YS, et al. (2001). Effects of iontophoretically applied substance P, calcitonin gene-related peptide on excitability of dorsal horn neurones in rats. *Yonsei Med J* 42(1): 74-83.
- Lester GE, Vanderwiel CJ, et al. (1982). Vitamin D deficiency in rats with normal serum calcium concentrations. *Proc Natl Acad Sci U S A* 79(15): 4791-4794.
- Levenson CW and Figueiroa SM (2008). Gestational vitamin D deficiency: long-term effects on the brain. *Nutr Rev* 66(12): 726-729.
- Levy J, Zuili I, et al. (1984). Induction of cytosolic receptors for 1 alpha, 25-dihydroxyvitamin D3 in the immature rat uterus by oestradiol. *J Endocrinol* 100(3): 265-269.

- Liel Y, Kraus S, et al. (1992). Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D receptors in osteoblast-like cells (ROS 17/2.8). *Endocrinology* 130(5): 2597-2601.
- Liel Y, Shany S, et al. (1999). Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. *Endocrinology* 140(1): 280-285.
- Linton SJ and Van Tulder MW (2001). Preventive interventions for back and neck pain problems: what is the evidence? *Spine* 26(7): 778-787.
- Loaiza LA, Yamaguchi S, et al. (2002). Vasodilatation of muscle microvessels induced by somatic afferent stimulation is mediated by calcitonin gene-related peptide release in the rat. *Neurosci Lett* 333(2): 136-140.
- Losel RM, Falkenstein E, et al. (2003). Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* 83(3): 965-1016.
- Lund B and Sorensen OH (1979). Measurement of circulating 1,25-dihydroxyvitamin D in man. Changes in serum concentrations during treatment with 1 alpha-hydroxycholecalciferol. *Acta Endocrinol (Copenh)* 91(2): 338-350.
- Macfarlane GJ, Palmer B, et al. (2005). An excess of widespread pain among South Asians: are low levels of vitamin D implicated? *Ann Rheum Dis* 64(8): 1217-1219.
- Maggi CA (1995). Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. *Prog Neurobiol* 45(1): 1-98.
- Mahonen A and Maenpaa PH (1994). Steroid hormone modulation of vitamin D receptor levels in human MG-63 osteosarcoma cells. *Biochem Biophys Res Commun* 205(2): 1179-1186.
- Mallon JP, Boris A, et al. (1981). Decrease in serum levels of 1,25-dihydroxycholecalciferol in rats and chickens fed a vitamin D-deficient diet. *J Nutr* 111(4): 665-667.
- Mascarenhas R and Mobarhan S (2004). Hypovitaminosis D-induced pain. *Nutr Rev* 62(9): 354-359.
- Masood H, Narang AP, et al. (1989). Persistent limb pain and raised serum alkaline phosphatase the earliest markers of subclinical hypovitaminosis D in Kashmir. *Indian J Physiol Pharmacol* 33(4): 259-261.
- Mastaglia FL and Hilton-Jones D (2007). Myopathies. Edinburgh ; New York, Elsevier.
- Mcbeth J, Pye SR, et al. (2010). Musculoskeletal pain is associated with very low levels of vitamin D in men: results from the European Male Ageing Study. *Ann Rheum Dis* 69(8): 1448-1452.

- Mccollum EV, Simmonds N, et al. (1922). An experimental demonstration of the existence of a vitamin which promotes calcium deposition. *J Biol Chem* 53: 293-298.
- Mccracken LM, Matthews AK, et al. (2001). A comparison of blacks and whites seeking treatment for chronic pain. *Clin J Pain* 17(3): 249-255.
- Mccracken LM and Turk DC (2002). Behavioral and cognitive-behavioral treatment for chronic pain: outcome, predictors of outcome, and treatment process. *Spine (Phila Pa 1976)* 27(22): 2564-2573.
- Meyer RA, Raja SN, et al. (1985). Coupling of action potential activity between unmyelinated fibers in the peripheral nerve of monkey. *Science* 227(4683): 184-187.
- Miller LK and Irving L (1962). Local reactions to air cooling in an Eskimo population. *J Appl Physiol* 17: 449-455.
- Mizwicki MT and Norman AW (2009). The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. *Sci Signal* 2(75): re4.
- Morales L, Pans S, et al. (2006). Debilitating musculoskeletal pain and stiffness with letrozole and exemestane: associated tenosynovial changes on magnetic resonance imaging. *Breast Cancer Res Treat*.
- Mouridsen HT (2006). Incidence and management of side effects associated with aromatase inhibitors in the adjuvant treatment of breast cancer in postmenopausal women. *Curr Med Res Opin* 22(8): 1609-1621.
- Mowa CN, Usip S, et al. (2003). The effects of pregnancy and estrogen on the expression of calcitonin gene-related peptide (CGRP) in the uterine cervix, dorsal root ganglia and spinal cord. *Peptides* 24(8): 1163-1174.
- Muller KA, Ryals JM, et al. (2008). Abnormal muscle spindle innervation and large-fiber neuropathy in diabetic mice. *Diabetes* 57(6): 1693-1701.
- Myhr KM (2009). Vitamin D treatment in multiple sclerosis. *J Neurol Sci* 286(1-2): 104-108.
- Nangia AK, Butcher JL, et al. (1998). Association of vitamin D receptors with the nuclear matrix of human and rat genitourinary tissues. *J Steroid Biochem Mol Biol* 66(4): 241-246.
- Nardone A, Galante M, et al. (2007). Balance control in Sensory Neuron Disease. *Clin Neurophysiol* 118(3): 538-550.
- Naveh-Many T, Raue F, et al. (1992). Regulation of calcitonin gene expression by hypocalcemia, hypercalcemia, and vitamin D in the rat. *J Bone Miner Res* 7(10): 1233-1237.

- Naveh-Many T and Silver J (1988). Regulation of calcitonin gene transcription by vitamin D metabolites in vivo in the rat. *J Clin Invest* 81(1): 270-273.
- Naveilhan P, Neveu I, et al. (1993). Expression of 25(OH) vitamin D3 24-hydroxylase gene in glial cells. *Neuroreport* 5(3): 255-257.
- Need AG (2006). Bone resorption markers in vitamin D insufficiency. *Clin Chim Acta* 368(1-2): 48-52.
- Need AG, Horowitz M, et al. (2000). Vitamin D status: effects on parathyroid hormone and 1, 25-dihydroxyvitamin D in postmenopausal women. *Am J Clin Nutr* 71(6): 1577-1581.
- Need AG, Morris HA, et al. (1993). Effects of skin thickness, age, body fat, and sunlight on serum 25-hydroxyvitamin D. *Am J Clin Nutr* 58(6): 882-885.
- Nelson ME, Meredith CN, et al. (1988). Hormone and bone mineral status in endurance-trained and sedentary postmenopausal women. *J Clin Endocrinol Metab* 66(5): 927-933.
- Neveu I, Naveilhan P, et al. (1994). 1,25-dihydroxyvitamin D3 regulates the synthesis of nerve growth factor in primary cultures of glial cells. *Brain Res Mol Brain Res* 24(1-4): 70-76.
- Newmark HL and Newmark J (2007). Vitamin D and Parkinson's disease--a hypothesis. *Mov Disord* 22(4): 461-468.
- Norman AW, Okamura WH, et al. (1997). Comparison of 6-s-cis- and 6-s-trans-locked analogs of 1alpha,25-dihydroxyvitamin D3 indicates that the 6-s-cis conformation is preferred for rapid nongenomic biological responses and that neither 6-s-cis- nor 6-s-trans-locked analogs are preferred for genomic biological responses. *Mol Endocrinol* 11(10): 1518-1531.
- Omdahl JL, Bobrovnikova EV, et al. (2003). Expression, structure-function, and molecular modeling of vitamin D P450s. *J Cell Biochem* 88(2): 356-362.
- Ooms ME, Roos JC, et al. (1995). Prevention of bone loss by vitamin D supplementation in elderly women: a randomized double-blind trial. *J Clin Endocrinol Metab* 80(4): 1052-1058.
- Pal BR, Marshall T, et al. (2003). Distribution analysis of vitamin D highlights differences in population subgroups: preliminary observations from a pilot study in UK adults. *J Endocrinol* 179(1): 119-129.
- Palmer KT, Syddall H, et al. (2003). Smoking and musculoskeletal disorders: findings from a British national survey. *Ann Rheum Dis* 62(1): 33-36.

- Pang X, Marchand J, et al. (1995). Increased number of substance P positive nerve fibres in interstitial cystitis. *Br J Urol* 75(6): 744-750.
- Perez-Fernandez R, Alonso M, et al. (1997). Vitamin D receptor gene expression in human pituitary gland. *Life Sci* 60(1): 35-42.
- Plotnikoff GA and Quigley JM (2003). Prevalence of severe hypovitaminosis D in patients with persistent, nonspecific musculoskeletal pain. *Mayo Clin Proc* 78(12): 1463-1470.
- Prufer K, Racz A, et al. (2000). Dimerization with retinoid X receptors promotes nuclear localization and subnuclear targeting of vitamin D receptors. *J Biol Chem* 275(52): 41114-41123.
- Prufer K, Veenstra TD, et al. (1999). Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. *J Chem Neuroanat* 16(2): 135-145.
- Racz A and Barsony J (1999). Hormone-dependent translocation of vitamin D receptors is linked to transactivation. *J Biol Chem* 274(27): 19352-19360.
- Randolph JF, Jr., Sowers M, et al. (2004). Change in estradiol and follicle-stimulating hormone across the early menopausal transition: effects of ethnicity and age. *J Clin Endocrinol Metab* 89(4): 1555-1561.
- Raue F, Deutschle I, et al. (1984). Reversible diminished calcitonin secretion in the rat during chronic hypercalcemia. *Endocrinology* 115(6): 2362-2367.
- Raue F, Deutschle I, et al. (1983). Acute effect of 1,25-dihydroxy-vitamin D3 on calcitonin secretion in rats. *Horm Metab Res* 15(4): 208-209.
- Reinert A, Kaske A, et al. (1998). Inflammation-induced increase in the density of neuropeptide-immunoreactive nerve endings in rat skeletal muscle. *Exp Brain Res* 121(2): 174-180.
- Reynolds ML and Fitzgerald M (1995). Long-term sensory hyperinnervation following neonatal skin wounds. *J Comp Neurol* 358(4): 487-498.
- Richardson JD and Vasko MR (2002). Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther* 302(3): 839-845.
- Rollman GB and Lautenbacher S (2001). Sex differences in musculoskeletal pain. *Clin J Pain* 17(1): 20-24.
- Ruscheweyh R, Forsthuber L, et al. (2007). Modification of classical neurochemical markers in identified primary afferent neurons with A-beta-, A-delta-, and C-fibers after chronic constriction injury in mice. *J Comp Neurol* 502(2): 325-336.

- Sakaki T, Kagawa N, et al. (2005). Metabolism of vitamin D3 by cytochromes P450. *Front Biosci* 10: 119-134.
- Salaffi F, De Angelis R, et al. (2005). Health-related quality of life in multiple musculoskeletal conditions: a cross-sectional population based epidemiological study. II. The MAPPING study. *Clin Exp Rheumatol* 23(6): 829-839.
- Sansone RA and Sansone LA (2009). Tramadol: seizures, serotonin syndrome, and coadministered antidepressants. *Psychiatry (Edgmont)* 6(4): 17-21.
- Schochat T and Raspe H (2003). Elements of fibromyalgia in an open population. *Rheumatology (Oxford)* 42(7): 829-835.
- Schott GD and Wills MR (1976). Muscle weakness in osteomalacia. *Lancet* 1(7960): 626-629.
- Schubert TE, Weidler C, et al. (2005). Achilles tendinosis is associated with sprouting of substance P positive nerve fibres. *Ann Rheum Dis* 64(7): 1083-1086.
- Schwartz B, Smirnoff P, et al. (2000). Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Mol Cell Biochem* 203(1-2): 87-93.
- Scragg R, Sowers M, et al. (2007). Serum 25-hydroxyvitamin D, ethnicity, and blood pressure in the Third National Health and Nutrition Examination Survey. *Am J Hypertens* 20(7): 713-719.
- Scroggs RS and Fox AP (1992). Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. *J Physiol* 445: 639-658.
- Shaffer PL and Gewirth DT (2004). Vitamin D receptor-DNA interactions. *Vitam Horm* 68: 257-273.
- Sharma NK, Ryals JM, et al. (2009). Acidic saline-induced primary and secondary mechanical hyperalgesia in mice. *J Pain* 10(12): 1231-1241.
- Shaw NJ and Pal BR (2002). Vitamin D deficiency in UK Asian families: activating a new concern. *Arch Dis Child* 86(3): 147-149.
- Shinoda M, Honda T, et al. (2003). Nerve terminals extend into the temporomandibular joint of adjuvant arthritic rats. *Eur J Pain* 7(6): 493-505.
- Sievert LL and Goode-Null SK (2005). Musculoskeletal pain among women of menopausal age in Puebla, Mexico. *J Cross Cult Gerontol* 20(2): 127-140.
- Skyba DA, Radhakrishnan R, et al. (2005). Characterization of a method for measuring primary hyperalgesia of deep somatic tissue. *J Pain* 6(1): 41-47.

- Sohrabji F, Miranda RC, et al. (1994). Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. *J Neurosci* 14(2): 459-471.
- Sowers MR, Wallace RB, et al. (1986). Parameters related to 25-OH-D levels in a population-based study of women. *Am J Clin Nutr* 43(4): 621-628.
- Steinman JL, Carlton SM, et al. (1992). The segmental distribution of afferent fibers from the vaginal cervix and hypogastric nerve in rats. *Brain Res* 575(1): 25-31.
- Stewart WF, Ricci JA, et al. (2003). Lost productive time and cost due to common pain conditions in the US workforce. *Jama* 290(18): 2443-2454.
- Tague SE and Smith PG (2010). Vitamin D receptor and enzyme expression in dorsal root ganglia of adult female rats: Modulation by ovarian hormones. *J Chem Neuroanat*.
- Takahashi Y, Chiba T, et al. (2003). Dermatomes and the central organization of dermatomes and body surface regions in the spinal cord dorsal horn in rats. *J Comp Neurol* 462(1): 29-41.
- Tanaka K, Matsugami T, et al. (2002). The origin of sensory innervation of the peritoneum in the rat. *Anat Embryol (Berl)* 205(4): 307-313.
- Tanaka Y, Castillo L, et al. (1978). Synergistic effect of progesterone, testosterone, and estradiol in the stimulation of chick renal 25-hydroxyvitamin D3-1alpha-hydroxylase. *Endocrinology* 103(6): 2035-2039.
- Tandeter H, Grynbaum M, et al. (2009). Serum 25-OH vitamin D levels in patients with fibromyalgia. *Isr Med Assoc J* 11(6): 339-342.
- Tishkoff DX, Nibbelink KA, et al. (2008). Functional vitamin D receptor (VDR) in the t-tubules of cardiac myocytes: VDR knockout cardiomyocyte contractility. *Endocrinology* 149(2): 558-564.
- Tiwari S, Gupta SK, et al. (2002). Short-term androgen deprivation does not alter CaR and VDR mRNA expression in duodenal mucosa in male rats. *Indian J Exp Biol* 40(7): 780-784.
- Tramer MR, Moore RA, et al. (2000). Quantitative estimation of rare adverse events which follow a biological progression: a new model applied to chronic NSAID use. *Pain* 85(1-2): 169-182.
- Tseng KW, Chau YP, et al. (2008). Abnormal cellular translocation of alpha-internexin in spinal motor neurons of Dystonia musculorum mice. *J Comp Neurol* 507(1): 1053-1064.
- Uhland AM, Kwiecinski GG, et al. (1992). Normalization of serum calcium restores fertility in vitamin D-deficient male rats. *J Nutr* 122(6): 1338-1344.

- Van Hoof HJ, Van Der Mooren MJ, et al. (1994). Hormone replacement therapy increases serum 1,25-dihydroxyvitamin D: A 2-year prospective study. *Calcif Tissue Int* 55(6): 417-419.
- Van Hoof HJ, Van Der Mooren MJ, et al. (1999). Female sex hormone replacement therapy increases serum free 1,25-dihydroxyvitamin D3: a 1-year prospective study. *Clin Endocrinol (Oxf)* 50(4): 511-516.
- Veenstra TD, Prufer K, et al. (1998). 1,25-Dihydroxyvitamin D3 receptors in the central nervous system of the rat embryo. *Brain Res* 804(2): 193-205.
- Vieth R, Ladak Y, et al. (2003). Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggest a different reason why older adults require more vitamin D. *J Clin Endocrinol Metab* 88(1): 185-191.
- Walbert T, Jirikowski GF, et al. (2001). Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the limbic system of the rat. *Horm Metab Res* 33(9): 525-531.
- Walters MR (1981). An estrogen-stimulated 1,25-dihydroxyvitamin D3 receptor in rat uterus. *Biochem Biophys Res Commun* 103(2): 721-726.
- Waltman NL, Ott CD, et al. (2009). Vitamin D insufficiency and musculoskeletal symptoms in breast cancer survivors on aromatase inhibitor therapy. *Cancer Nurs* 32(2): 143-150.
- Wang Y, Becklund BR, et al. (2010). Identification of a highly specific and versatile vitamin D receptor antibody. *Arch Biochem Biophys* 494(2): 166-177.
- Warner AE and Arnsperger SA (2008). Diffuse musculoskeletal pain is not associated with low vitamin D levels or improved by treatment with vitamin D. *J Clin Rheumatol* 14(1): 12-16.
- Weishaar RE and Simpson RU (1987). Involvement of vitamin D3 with cardiovascular function. II. Direct and indirect effects. *Am J Physiol* 253(6 Pt 1): E675-683.
- Weishaar RE and Simpson RU (1987). Vitamin D3 and cardiovascular function in rats. *J Clin Invest* 79(6): 1706-1712.
- White KP, Speechley M, et al. (1999). The London Fibromyalgia Epidemiology Study: the prevalence of fibromyalgia syndrome in London, Ontario. *J Rheumatol* 26(7): 1570-1576.
- Wietzke JA, Ward EC, et al. (2005). Regulation of the human vitamin D3 receptor promoter in breast cancer cells is mediated through Sp1 sites. *Mol Cell Endocrinol* 230(1-2): 59-68.
- Wolfe F, Ross K, et al. (1995). The prevalence and characteristics of fibromyalgia in the general population. *Arthritis Rheum* 38(1): 19-28.

- Woodhouse PR, Khaw KT, et al. (1993). Seasonal variation of blood pressure and its relationship to ambient temperature in an elderly population. *J Hypertens* 11(11): 1267-1274.
- Wortsman J, Matsuoka LY, et al. (2000). Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr* 72(3): 690-693.
- Xiao WH and Bennett GJ (2008). C-fiber spontaneous discharge evoked by chronic inflammation is suppressed by a long-term infusion of lidocaine yielding nanogram per milliliter plasma levels. *Pain* 137(1): 218-228.
- Yasuhara O, Aimi Y, et al. (2008). Distribution of a splice variant of choline acetyltransferase in the trigeminal ganglion and brainstem of the rat: comparison with calcitonin gene-related peptide and substance P. *J Comp Neurol* 509(4): 436-448.
- Yetley EA (2008). Assessing the vitamin D status of the US population. *Am J Clin Nutr* 88(2): 558S-564S.
- Yunus MB, Arslan S, et al. (2002). Relationship between body mass index and fibromyalgia features. *Scand J Rheumatol* 31(1): 27-31.
- Yunus MB, Arslan S, et al. (2002). Relationship between fibromyalgia features and smoking. *Scand J Rheumatol* 31(5): 301-305.
- Zanello LP and Norman AW (2004). Rapid modulation of osteoblast ion channel responses by 1 α ,25(OH) $_2$ -vitamin D $_3$ requires the presence of a functional vitamin D nuclear receptor. *Proc Natl Acad Sci U S A* 101(6): 1589-1594.
- Zehnder D, Bland R, et al. (2001). Extrarenal expression of 25-hydroxyvitamin d(3)-1 α -hydroxylase. *J Clin Endocrinol Metab* 86(2): 888-894.